Ecology of parasite-vector interactions

edited by:

Willem Takken and Constantianus J.M. Koenraadt

Ecology and control of vector-borne diseases
Volume 3

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Ecology and control of vector-borne diseases

In the past century, many advances were made in the control of vector-borne diseases. Malaria disappeared from the northern hemisphere, diseases such as typhus, *Bartonella* and yellow fever were seriously reduced in prevalence and in many countries effective methods of disease control contributed to a greatly reduced incidence of such diseases. Most of these advances were beneficial to the industrialised world, whereas underdeveloped countries continued to suffer much as before. Indeed, several diseases such as malaria, Rift Valley fever and African sleeping sickness are still highly prevalent in parts of the tropics. 'New' vector-borne diseases such as dengue, chikungunya fever and West Nile fever, have emerged and are invading previously disease-free regions. The discovery of new drugs and vaccines has made great advances and allows for the effective treatment and control of many diseases. In contrast, vector control has lagged behind in development, even though it is realised that effective vector control would allow for an immediate interruption of the transmission of disease, and aid in disease control and eradication. In the last decade new initiatives on vector control have been undertaken, leading to a rapid development of effective and lasting methods of vector control. For example, the Roll Back Malaria control programme of the World Health Organization has led to significant reductions in malaria in many countries. In order to achieve further advances, however, additional tools are required. The development of molecular genetics has provided new insight in vector biology and behaviour, which is being used for developing new strategies of vector control. Advances in geographic information systems allow for precision targeting of interventions. The collective information on new developments in Vector Ecology and Control for Vector-borne Diseases is scattered over numerous periodicals and electronic databases. This book series intends to bring together this information in sequential volumes arranged around selected themes that are currently of interest. Forthcoming themes will include 'Recent advances in biological control of mosquitoes', 'Transgenic tools for vector management' and 'Integrated management of vectors of livestock diseases', but also fundamental biological topics such as 'Mating behaviour of disease vectors', 'Oviposition behaviour of disease vectors' and 'Reproductive strategies of disease vectors'. Other topics will be added as perceived relevant.

Willem Takken is the senior editor of the series. Each volume will be co-edited by a guest editor, which in Volume 3 is Sander Koenraadt. The editors of the current volume are well-known experts in the field of Medical and Veterinary Entomology, and have experience from field work in the tropics and ecological studies in laboratory and field. Willem Takken is professor in Medical and Veterinary Entomology at Wageningen University. Sander Koenraadt is an assistant professor in Vector Ecology at Wageningen University. Both editors collaborate in several research programmes, and consider dissemination of research results to fellow scientists as well as the public at large as an important component of their work.

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Preface

The current book is the 3rd volume of the series Ecology and Control of Vector-borne Diseases. The series is intended to cover a wide series of topics that concern vector-borne diseases, from fundamental research to control. By focusing on specific subjects in each Volume, an in-depth and up to date information about these subjects is provided, which can help to gain a perspective of recent advances in knowledge and how this can be exploited for effective disease control.

When the series was launched in 2007 (ECVD Vol. 1), we could not imagine that vector-borne diseases would dominate the international scene at a scale as we have witnessed in the last few years. Following the 2006 outbreak of bluetongue virus in north-western Europe, outbreaks of other vector-borne diseases in Europe followed each other with increasing speed: chikungunya in Italy (2007) and then in France (2010), dengue in France (2010) and recently in Greece (2012) for the first time since the nineteen twenties, new strains of West Nile virus in southern Europe (2011, 2012) and Usutu virus advances in Germany (2012). In the USA, Florida has experienced serious outbreaks of dengue in 2010, while Texas recently experienced its worst epidemic of West Nile virus since the disease arrived in the USA in 1999. In the tropics, where spectacular advances have been made in the control of malaria, insecticide and drug resistance threaten to halt this process. Of equal concern is the global advance of dengue, which has become a disease of the urban environment. Not only do vector-borne diseases emerge and expand their geographical territories, also some of the vector species migrate across huge distances. *Aedes albopictus* is probably the best example of a highly successful global migrant, and is now likely to become established in Australia as well due to its highly competitive nature. In Europe, *Aedes japonicus* has become established in Switzerland, and small pockets of this species were found elsewhere on the continent as well.

The realisation that vector-borne diseases continue to be prevalent and are capable of rapidly invading novel geographic areas, is reason to continue investing in research on the basic biology of these diseases as well as in the development of preventive and effective control measures. Variation in parasite and vector genetics and ecological determinants provide challenges that need to be met. The current volume of ECVD focuses on the interaction between parasites and vectors. As several of the chapters will show, rapid advances in molecular genetics allow for some revision of our classical knowledge about vector-parasite interactions and provide insight in the intricate regulation of these interactions, notably the role of immune responses as well as endosymbionts. Better insight in these processes can provide the key to successful interruption of these interactions and hence to disease control.

Many scientists have contributed to this Volume, and we appreciate the time taken to complete these chapters, which show strong interlinkages about the subject of parasite-vector interactions. Each chapter was reviewed by independent reviewers, and subsequently adjusted as needed. We are grateful to the anonymous reviewers, as we realise that this takes time and effort. We thank Wageningen Academic Publishers for their patience and useful advice. We thank Hans Smid for once more having contributed to the cover design.

Wageningen, 22 October 2012

Willem Takken and Constantianus JM (Sander) Koenraadt

1. Introduction – who was there first?

Willem Takken and Constantianus J.M. Koenraadt

Abstract

The force of vector-borne disease transmission is greatly affected by interactive processes between parasites and their arthropod hosts. In recent years significant advances in knowledge about the mechanisms of these interactions have been made, notably concerning the impact of arthropod immune responses on parasite establishment and propagation in the arthropod host, genetic and phenotypic variation affecting these interactions, the impact of these interactions on parasite and arthropod fitness, and how environmental factors affect parasite transmission. The current volume of the Ecology and Control of Vector-Borne Diseases highlights significant and novel aspects of parasite-vector interactions and contributes to a better understanding of vector-borne disease transmission. Better insight in these interactive processes will be useful for studies on the epidemiology and control of vector-borne diseases and is expected to contribute to the development of novel intervention strategies.

Keywords: vector-borne disease, parasites, pathogens, interaction, genetics, immunity, transmission

Introduction

Vector-borne diseases are characterized by the fact that one organism is dependent for its existence on at least two other organisms, one vertebrate host and one arthropod host. Examples are the leishmaniases with rodents as the vertebrate reservoir and sandflies (Diptera: Phlebotominae) as the arthropod hosts, dengue with humans as the vertebrate reservoir and *Aedes* mosquitoes (Diptera: Culicidae) as arthropod hosts and African trypanosomiasis, with various mammalian species as reservoir hosts and tsetse flies (Diptera: Glossinidae) as arthropod hosts. Historically, much attention has been paid to the parasite-vertebrate host interaction, as knowledge about this association is essential for understanding of the disease and contains clues for treatment. This includes research on the biology of parasites, the onset and progress of clinical disease, and preventive and curative interventions. Conversely, the parasite-arthropod interaction has received far less attention, presumably because it was originally assumed that passage of the parasite through the arthropod would not affect the latter. With hindsight, this was a remarkable attitude, as it was realized soon after the discovery of the role of arthropods as vectors that the parasites would require nutrients from their hosts for survival and reproduction (reviewed by Hurd 1990).

The realization that parasites can manipulate their arthropod hosts, or that the arthropods elicit effective immune responses against invading parasites coupled with rapidly-advancing technologies for parasite detection and identification (Sim *et al.* 2009, Valkiunas *et al.* 2008), has led to a growing body of research on parasite-vector interactions that has assisted greatly in our understanding of the biology of vector-borne diseases. For example, the development of real-time quantitative nucleic acid sequence-based amplification (QT-NASBA) techniques has led to the discovery of sub-microscopic infectious *Plasmodium* stages in the human host, suggesting that malaria parasite transmission may occur at a much greater scale than was believed hitherto (Schneider *et al.* 2007). Advances in immunology allow for a detailed understanding of the infectious route of *Plasmodium* spp. in anopheline mosquitoes (Chapter 2, Cirimotich *et al.* 2010). Molecular tools revealed the highly complex interactions between trypanosome parasites and their tsetse fly hosts, showing the effect of symbiotic interactions on successful parasite establishment following

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an infectious blood meal, as well as parasite multiplication and passage to the salivary gland (Aksoy and Rio 2005). These advances affect not only the true parasite-vector interactions, but also those involving true pathogens such as viruses, bacteria and fungi. Recent studies demonstrated the role of cellular mechanisms of *Ixodes ricinus* (L.) ticks (Acari: Ixodidae) in the establishment and subsequent multiplication of *Borrelia burgdorferi* spp. in the tick host (Schuijt *et al.* 2011), or of the impact of the mosquito host on the replication of dengue virus (Sim and Dimopoulos 2010). New insights about parasite-host interactions are not limited to direct interactions at the dual level of parasite and arthropod host, but also about environmental factors such as temperature, humidity and symbionts. For example, it was recently shown that daily temperature fluctuations greatly affect the transmission of *Plasmodium* (Chapter 5, Paaijmans *et al.* 2012) as well as dengue virus (Lambrechts *et al.* 2011) by their respective mosquito vectors. The relevant role of endosymbionts in the regulation of parasite-vector interactions is becoming increasingly realized, especially as the symbionts not only provide essential nutrients to the arthropod host, but also affect immune responses as well as the fitness of the parasite (Chapter 2, Hughes *et al.* 2011, McMeniman *et al.* 2009, Pinto *et al.* 2012). Recently, it was discovered that *Chromobacterium* spp. present in the midgut of mosquitoes possibly release anti-pathogenic agents that kill *Plasmodium* parasites as well as dengue virus (G. Dimopoulos, personal communication) and it is likely that microbial-vector interactions affect the successful establishment of parasites in the vector in ways that are not yet understood.

These examples are only a fraction of the vast amount of knowledge on parasite-vector interactions that has emerged in recent years. Such knowledge is likely to affect our understanding of vector-borne disease epidemiology, and can potentially be used for more effective interventions aimed at the control of vector-borne diseases. The revelation of malaria hot spots (Chapter 11, Bousema *et al.* 2012)) was only made possible through these advancements and provides new opportunities for more effective, targeted malaria control. The insertion of *Wolbachia pipientis* Hertig in *Aedes aegypti* (L.) has revealed a novel implementation of endosymbionts for the control of dengue virus (Frentiu *et al.* 2010) and will shortly be tested in dengue-endemic areas (S. O'Neill, personal communication).

The chapters in this third volume of ECVD highlight current advances in research on parasite-vector interactions, and provide proof that such advances are essential for the improvement of current disease control strategies. With the rapid advancement of insecticide resistance against malaria vectors (Asidi *et al.* 2012, Ranson *et al.* 2011) coupled with increasing drug resistance and the apparent spread of vector-borne diseases associated with environmental change (see Takken and Knols 2007), effective tools for vector-borne disease control are urgently needed, and studies that lead to better understanding of parasite-vector interactions will contribute to achieve that goal.

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Fundamental aspects of vector-parasite interactions

2. Impact of transgenic immune deployment on mosquito fitness

Andrew D. Pike, Chris M. Cirimotich and George Dimopoulos

Abstract

Mosquitoes are the vectors of pathogens causing numerous human diseases, including dengue and malaria. Due to increases in drug resistance among pathogens or the lack of effective treatments for these diseases and increasing insecticide resistance among mosquito populations, new methods of control are urgently needed to limit the morbidity and mortality caused by vector-borne diseases. Mosquitoes possess an innate immune system capable of limiting infection with human pathogens, and the creation and deployment of transgenic mosquitoes with an enhanced immune system has been suggested as a novel means to reduce mosquito vector competence. However, activation of the immune system is often associated with a cost to the host, which could limit the ability of the transgenic insects to replace their wild-type conspecifics. Here, we discuss recent research into the effects of increased immune deployment and insect transgenesis on the fitness of the mosquitoes.

Keywords: insect transgenesis, fitness, innate immunity, vector-borne disease, vectorial capacity, *Drosophila*, *Anopheles*

Introduction

Mosquitoes serve as vectors for numerous viral, filarial and protozoan pathogens of great public health importance in both humans and other animals. Principal among these agents are the *Plasmodium* parasites responsible for malaria and the viruses responsible for dengue fever and dengue haemorrhagic fever/dengue shock syndrome. Mosquitoes become infected with these pathogens when acquiring a blood meal from an infected vertebrate host and, following an incubation period during which the pathogen develops or replicates within the vector mosquito, they transmit the pathogens to a susceptible host during a subsequent blood meal.

Many factors contribute to the ability of a mosquito to successfully transmit a pathogen and to the efficiency of disease transmission, referred to as the vectorial capacity. The inherent capability of a mosquito to transmit the pathogen, or vector competence, is determined by genetic components of the mosquito and pathogen as well as by environmental components, including temperature. Other variables involved in vectorial capacity include the vector population density, the extrinsic incubation period required for vectors to become infectious and the daily survival rate of competent vectors. A mathematical model of vectorial capacity was first described by Ross (1911) and later refined by MacDonald (1957) and Smith and MacKenzie (2004). The full equation is given by

$$C = \frac{\beta m a^2 p^n}{-\ln p},$$

where C is the vectorial capacity, β is the vector competence, m is the density of the vector population, a is the daily biting rate, n is the extrinsic incubation period of the pathogen and p is the daily survival rate. A change in any one of these variables can greatly affect the overall vectorial capacity for a specific pathogen and alter the persistence of the disease.

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The advent of molecular biology, genomics and functional genomics has provided unprecedented opportunities to elucidate the complex interactions that take place between the mosquito vector and the pathogens it transmits. These technologies have led to significant advances in our understanding of how the mosquito's innate immune system is actively involved in killing large fractions of these human pathogens, sometimes rendering the mosquito vector completely refractory to infection. The progress made in basic research, together with the development of mosquito transgenic methodologies, has opened the way for the development of novel disease control strategies that are based on blocking pathogen transmission in genetically modified immune-enhanced mosquitoes. However, despite the ongoing development of powerful genetic drive systems, a potential bottleneck in the course of successful deployment of genetically modified pathogen-immune mosquitoes is the possible impact of the immune transgene on mosquito fitness. This chapter will address our current understanding of the interactions between the insect immune system and the organism's fitness and how that interplay might influence the use of genetically modified pathogen-immune mosquitoes for vector-borne disease control.

The mosquito innate immune system

Infection of a mosquito with a parasite or virus has a profound effect on the transcriptional repertoire of the mosquito. Hundreds of genes are regulated and implicated during infection, especially those encoding factors involved in the mosquito innate immune response (Dong *et al.* 2006, Xi *et al.* 2008). Mosquito genetics play a crucial role in vector competence, and especially in the inherent ability of the mosquito to mount an effective neutralizing immune response against the invading pathogen. Unlike humans and other mammals, mosquitoes do not have genes for the production of antibodies and other molecules of the adaptive immune response. Instead, the mosquito's innate immune system directly responds to and combats pathogens upon challenge. Pattern recognition receptors (PRRs) on the surface of immune-competent cells, or circulating in the haemolymph, bind to specific pathogen-associated molecular patterns (PAMPs), triggering a series of reactions that culminate in the expression of anti-pathogen effector molecules. The ultimate result of immune pathway activation is an up-regulation of specific gene expression that is PAMP- and pathway-dependent. These immune effector genes form an important line of defence for the mosquito against a variety of invading pathogens. PRRs can also directly activate immune defence mechanisms such as phagocytosis and complement-like killing mechanisms, independent of the intracellular immune signalling pathways.

Various cellular and humoral factors in the mosquito haemolymph play a significant role in the response to microbial challenge. Circulating immune-competent cells, known as haemocytes, phagocytose and encapsulate foreign particles and pathogens. Simultaneously, serine protease cascades activate enzymes that generate melanin and free radicals, which are responsible for killing microbes during humoral responses. These effectors create a series of barriers that a pathogen must surmount before the mosquito becomes infectious, and an increase in any of these anti-pathogen factors can greatly reduce the vector competence of the mosquito.

A number of immune signalling pathways regulate anti-pathogen immunity in mosquitoes. With the advent of whole-genome sequencing projects over the past decade (Holt *et al.* 2002, Nene *et al.* 2007), the three major known immune signalling pathways (Toll, IMD, and Jak/Stat) that were originally described in *Drosophila* or mammals have been identified through orthology in mosquitoes (Christophides *et al.* 2001).

The Toll pathway has been implicated in the mosquito defence against fungal, bacterial, parasitic and viral infections (Antonova *et al.* 2009, Shin *et al.* 2005, Xi *et al.* 2008). PAMP recognition by Toll pathway PRRs is well documented, but the underlying mechanism is still unresolved. The *Drosophila* genome encodes two distinct Toll pathway-regulated transcription factors, *Dif* and *Dorsal*, which mediate immune and developmental gene expression, respectively. The *Aedes aegypti* (L.) genome also encodes two distinct Toll pathway transcription factors (REL1A and REL1B), while the *Anopheles gambiae* Giles genome encodes a single factor (REL1/GAMBIF1) (Barillas-Mury *et al.* 1996, Shin *et al.* 2002).

It has been shown through transient activation of the Toll pathway via silencing of the negative regulator *cactus* that Rel1-transcribed effector molecules are critical for the *Ae. aegypti* defence against dengue viruses (Ramirez and Dimopoulos 2010, Xi *et al.* 2008) and the *An. gambiae* defence against rodent malaria parasites (Frolet *et al.* 2006, Garver *et al.* 2009, Meister *et al.* 2005, Zou *et al.* 2008). Frolet *et al.* (2006) and Garver *et al.* (2009) used this transient immune stimulation to show that Toll pathway activation decreases the *Plasmodium berghei* parasite burden, whereas depletion of the Rel1 transcription factor increases infection levels in mosquito midguts. Frolet *et al.* (2006) suggest that Toll pathway-regulated effector molecules are constantly in circulation and can immediately attack an invading pathogen. Transcriptional activation subsequent to pathogen challenge is then used to replenish molecules used during the initial insult (Frolet *et al.* 2006). However, Toll pathway-mediated killing of parasites may not be relevant to all parasite species. *P. berghei* infection of *An. gambiae*, *Anopheles stephensi* Liston and *Anopheles albimanus* Wiedemman, as well as *Plasmodium gallinaceum* Brumpt infection of *Ae. aegypti* appear to be controlled through Toll pathway activation, while *Plasmodium falciparum* infection of various anopheline mosquitoes is affected to a lesser degree by the Toll pathway (Garver *et al.* 2009, Zou *et al.* 2008).

Initiation of signalling through a second innate immune pathway, the immune deficiency (IMD) pathway, protects mosquitoes from infection with *Plasmodium*, especially the human malaria parasite *P. falciparum* (Dong *et al.* 2011, Garver *et al.* 2009, Meister *et al.* 2005, 2009, Mitri *et al.* 2009). Signalling events within this pathway culminate in the expression of various effector genes mediated by the Rel2 transcription factor. Basal levels of IMD pathway-mediated gene expression are constantly regulated by a shortened splice variant of Rel2, while the full-length isoform is continuously present in the cell cytoplasm, but inactive until immune stimulation occurs (Luna *et al.* 2006, Meister *et al.* 2005). Pathway activation stimulates the cleavage of the full-length isoform, exposing the nuclear localization signal and causing nuclear translocation of the transcription factor and a subsequent increase in the transcription of immune effectors. Garver *et al.* (2009) used transient depletion of the negative regulator *caspar*, and Dong *et al.* (2011) used transgenic over expression of Rel2, to show that induction of the IMD pathway renders mosquitoes nearly refractory to *P. falciparum* infection. Interestingly, both the Toll and IMD pathways are mosquito species-independent, in that multiple mosquito species use the same pathways to combat pathogens, but are *Plasmodium* species-dependent.

The third major immune pathway, the Jak/Stat pathway, is named for the kinases (Jak) and transcription factors (STAT) that control its activation. The pathway has antiviral activity in *Ae. aegypti* (Souza-Neto *et al.* 2009) and can control *Anopheles-Plasmodium* interactions during the later stages of infection (Gupta *et al.* 2009). Interestingly, in contrast to *An. gambiae*, the *Ae. aegypti* Jak/Stat pathway also controls *P. gallinaceum* infection at the early pre-oocyst stages (Zou *et al.* 2011). Two STAT transcription factors, STAT-A and STAT-B, have been identified in *An. gambiae*, while only one STAT is present in *Ae. aegypti*. In *An. gambiae*, STAT-B modulates the transcription of STAT-A, the ancestral transcription factor and predominant form in adult mosquitoes. Translocation

of STAT-A to the nucleus leads to up-regulation of anti-pathogen effector molecule expression. In experiments similar to those described above for the Toll and IMD pathways, activation of the Jak/STAT pathway via depletion of the negative regulator *SOCS* decreases the density of *P. berghei* late oocysts, indicating that the pathway is important for anti-*Plasmodium* responses in *Anopheles* malaria vectors (Gupta *et al.* 2009).

Activation of any innate immune pathway leads to an increase in the production of various anti-pathogen molecules. A large number of anti-*Plasmodium* effector molecules have been identified, including leucine-rich repeat domain-containing proteins, fibrinogen-related proteins, C-type lectins, and others (reviewed in Cirimotich *et al.* 2010). Of particular interest is the thioester-containing protein TEP1, which has been shown to be crucial for mosquito defence against *Plasmodium* parasites (Levashina *et al.* 2001). TEP1, a homolog to the vertebrate complement system molecule C3, is constitutively secreted by haemocytes into the mosquito haemolymph, allowing it to interact with pathogens soon after they infect the mosquito (Levashina *et al.* 2001). Once a pathogen is detected, TEP1 binds to the surface of the invading microbe and promotes phagocytosis and, therefore, clearance of the intruder (Moita *et al.* 2005). TEP1 expression is induced in response to both Toll and IMD pathway activation, reflecting the molecule's importance in mosquito innate immune responses (Blandin *et al.* 2004, Garver *et al.* 2009, Levashina *et al.* 2001).

The dissection of the mosquito immune response to human pathogens has led to the discovery of immune pathway factors and downstream anti-pathogen effectors that can potentially be used to render the mosquito resistant to these infections through transgenic tissue- and infection stage-specific over-expression.

Mosquito transgenesis

The introduction of novel genetic elements into mosquito genomes has become a powerful approach for the study of mosquito immunity and has the potential to be used for future control of mosquito populations and to reduce the vectorial capacity of mosquitoes for human pathogens. In transgenesis, a mobile DNA element is used to introduce a gene of interest into the mosquito germline. This gene of interest is placed under the control of a specific promoter, which determines the tissue specificity and temporal expression of the transgene making it possible to express the gene with temporal and spatial specificity, or only when induced and only in certain tissues. Tools and methodologies for the genetic modification of *Anopheles* and *Aedes* mosquitoes have been developed and widely used to study various aspects of the vectors' biology. Successful transformation of mosquitoes was first achieved in *Ae. aegypti* (Coates *et al.* 1998, Jasinskiene *et al.* 1998) and soon followed by *An. stephensi* (Catteruccia *et al.* 2000), *An. gambiae* (Grossman *et al.* 2001) and *An. albimanus* (Perera *et al.* 2002), leading to the creation of many different strains of transgenic mosquitoes in each of these species.

In *Aedes* mosquitoes, transgenesis has been successfully used to identify Rel1-driven gene expression as a major component of anti-fungal immunity (Bian *et al.* 2005). It has also been used to show that Rel2-driven gene expression provides a defense against systemic bacterial challenge and *P. gallinaceum* infection (Antonova *et al.* 2009, Shin *et al.* 2003) and that RNA interference is crucial for antiviral defence (Khoo *et al.* 2010). Transgenesis has been utilized to study the innate immune pathways of *Anopheles* mosquitoes as well, and to express heterologous genes for the purpose of altering vector competence in both *Aedes* and *Anopheles* mosquitoes (reviewed in Cirimotich *et al.* 2011, Dong *et al.* 2011).

In order to affect the mosquito's vectorial capacity for a given pathogen, transgene expression must be driven in a relevant tissue, for instance the midgut, and at the appropriate time, i.e. when the pathogen has invaded that particular tissue. Midgut-, fat body- and salivary gland-specific promoters have been utilized to decrease vector competence in transgenic mosquitoes (Dong *et al.* 2011, Franz *et al.* 2006, Isaacs *et al.* 2011, Mathur *et al.* 2010). Mating of separate transgenic lines may eventually be used as a strategy to induce transgene expression in a single mosquito at multiple time points and locations, increasing the chances that pathogen development will be negatively affected and minimizing the possibility that the pathogens will be able to evade the immune response.

The implementation of transgenic technologies that utilize the mosquito innate immune system to combat vector-borne disease can largely be achieved in three ways: (1) over-expression of a pathway activator, such as a NF-κB transcription factor, to turn on the expression of anti-pathogen molecules; (2) depletion of negative regulators of a pathway through the expression of a hairpin transgene, again activating that specific pathway; and (3) over-expression of immune genes/effector molecules that directly affect the pathogen. Each approach has advantages and disadvantages, but regardless of the mechanism, the end result is a less suitable host environment for pathogen development.

The first and third strategies have previously been used experimentally in both *Ae. aegypti* and *An. stephensi* to demonstrate that this principle may eventually be applied to the engineering of pathogen-resistant mosquito populations (Antonova *et al.* 2009, Dong *et al.* 2011, Kokoza *et al.* 2010). As mentioned above, Rel2 has been over-expressed in *Ae. aegypti* in order to impede the development of *P. gallinaceum* parasites (Antonova et al, 2009). When Rel2 is expressed under the control of the vitellogenin promoter, which is inducible in the fat body of the mosquito upon blood-feeding, the transcription of a number of antimicrobial peptides (AMPs) is induced. These transgenic mosquitoes are more resistant than non-engineered mosquitoes to the establishment of *P. gallinaceum* infection in the midgut and sporozoite production in the haemolymph (Antonova *et al.* 2009). In follow-up studies, Kokoza *et al.* (2010) engineered *Ae. aegypti* mosquitoes to over-express the AMP genes *cecropin A* and *defensin A* directly, rather than inducing the entire Rel2-mediated pathway. Separate transgenic mosquito lines were engineered to induce either AMP gene singly or both together under the control of the vitellogenin promoter. Regardless of the configuration, transgenic expression of these genes decreased parasite development and completely abolished the vectorial capacity of the mosquitoes for parasite transmission, as measured by sporozoite production (Kokoza *et al.* 2010). Similar strategies have also been pursued in *An. stephensi* mosquitoes. Dong *et al.* (2011) created *An. stephensi* that overexpress Rel2 under the control of both the carboxypeptidase and vitellogenin promoters, leading to an increase in the ability of the mosquitoes to fight off both *Plasmodium* and bacterial infections.

Although candidate molecules exist for the development of mosquito refractoriness to various pathogens, the eventual release of genetically modified mosquitoes into the environment must coincide with the development of effective drive mechanisms to force the spread of the transgene into the native mosquito populations. The drive mechanism must have the power to drive the transgene to near-fixation in the native population and be sufficiently well-linked to the transgene to avoid separation from it. The current list of potential drive mechanisms includes transposable elements, homing endonuclease genes and the *Medea* system (reviewed in Cirimotich *et al.* 2011). The ability of a transgene to spread within mosquito populations will also be significantly affected by any fitness cost associated with transgene integration and expression.

Impact of insect immune system activation on its fitness

Although the immune system is vital to fighting off infection by various invading pathogens, long-term or constitutive over-expression of immune genes can negatively affect the host. This situation has been best described in autoimmune diseases of mammals, but it also occurs with the innate immune system of insects (DeVeale *et al.* 2004). Similarly, transgenic expression of heterologous genes or over-expression of endogenous genes can engender various fitness costs if the endogenous gene expression is interrupted or maintenance of transgene expression requires the use of necessary resources (Marrelli *et al.* 2006). In order to utilize transgenesis as a control mechanism for vector-borne disease in mosquitoes, the fitness effects of both the innate immune response to pathogens and the specific transgene expression must be assessed and understood. This will allow the creation of transgenic refractory vectors that can successfully compete with wild-type conspecifics and invade the natural population.

Fitness, most commonly indicated by the net reproductive rate, is considered to be the sum of many complex interactions that make it possible for an organism to reproduce. The two main components of fitness are lifespan and fecundity, or the ability to produce offspring successfully (Marrelli *et al.* 2006). Lifespan, or the length of time a disease vector is able to survive in its environment, is important because it determines the number of times an individual will be able to reproduce, whether the pathogen will have sufficient time to develop into an infectious stage and, finally, whether that individual is able to feed multiple times on blood in order to transmit the pathogen from one host to another. Daily survival is a particularly important component of vectorial capacity, since a vector that dies before the end of the pathogen's incubation period will be unable to transmit the pathogen. The significance of survival time is reflected in the fact that the daily survival rate is an exponential term in the vectorial capacity equation, indicating that even small changes in survival can lead to large changes in vectorial capacity for any pathogen transmitted by the mosquito (MacDonald 1957). Fecundity, usually measured by the number of viable offspring produced by an individual, is determined by both the number of times an organism is able to reproduce and the number of offspring that are produced during each reproductive cycle. If the fecundity and lifespan of a mosquito species decrease, the vectorial capacity will also be reduced, since the density of the vector will be reduced, and so will the chance that the mosquito will bite people. Both of these parameters are, in turn, affected by a large number of other factors, including the mosquito's ability to avoid predators, combat disease and allocate energetic resources.

Given the limited nutritional resources available to an organism during its life, an increase in the use of energy reserves for one purpose must lead to the reallocation of those resources from another area of activity. For instance, if an insect starts over-expressing certain immune effector genes in response to infection, or as a result of transgenesis, the raw materials and energy used to make those immune-related proteins must come from some other area. Similarly, if the insect has *X* arbitrary units of energy to use to produce all the proteins it needs to survive and reproduce, creating new proteins will utilize more of this energy. If the induction of the immune system causes more than *X* energy units to be used, the energy must be taken from that previously used to produce other proteins. This reallocation of resources could lead to a decrease in the ability of the insect to reproduce or a reduction in its lifespan, rendering it 'less fit' than an insect without immune activation. Infection of *Drosophila* as well as mosquitoes has been shown to alter the expression of hundreds of genes with diverse functions, indicating that the effects of infection are wide-ranging and not limited to immune deployment (Aguilar *et al.* 2005, De Gregorio *et al.* 2001, Dong *et al.* 2009). Because the factors that define fitness are only loosely defined, and because

the widespread effects of immune deployment have not yet been fully described, the effects of an increase in insect immune gene expression on fitness are difficult to predict.

Numerous studies have measured the effects of *Drosophila* immune activation on fly longevity. Many of these studies have been conducted by injecting wild-type and transgenic flies with various types of bacteria and measuring the fecundity or lifespan of the fly after infection. A negative effect of immune induction on fitness has generally been observed, suggesting a trade-off between fitness traits and the ability to deploy an effective immune response. For instance, wild-type flies infected with *Escherichia coli* Castellani and Chalmers or *Micrococcus luteus* (Schroeter) Cohn at various ages laid fewer eggs than their uninfected conspecifics, and interruption of the immune response by mutation of the genes *relish* and *imd-1*, components of the Toll and IMD pathways, respectively, alleviated these costs (Zerofsky *et al.* 2005). These findings show that infection, together with the activation of the immune system, can decrease a female fly's ability to reproduce. Induction of the Toll pathway also leads to a reallocation of resources in the fat body, suggesting a mechanism for this fitness cost and supporting the trade-off between immunity and reproduction (DiAngelo *et al.* 2009). These negative effects are significantly increased in food-limited environments, again adding support to the hypothesis of an energetic exchange between immune response and fitness. When flies were infected with the Gram-negative bacterium *Providencia rettgeri* Brenner, those provided with unlimited food exhibited no negative fitness effects of infection, while those with a limited food supply showed an inverse correlation between resistance and fecundity (McKean *et al.* 2008). Similarly, infection of wild-type flies with *Serratia marcescens* Bizio resulted in significantly reduced lifespans in both male and female flies, but only female flies experienced a decrease in fecundity, despite equivalent bacterial loads (Imroze and Prasad 2011). While the increase in mortality was the same in both sexes, the lack of an effect on male fecundity implies that the cost of infection and immune deployment depend on other energy expenditures. Because male flies require comparatively less energy to mate with females, there may be less of an effect on their fecundity than in females, which must invest a greater amount of energy into production of eggs. However, males must also spend a significant amount of energy on attracting a mate and may experience more mating competition, and several studies have observed that infection has a greater effect on the lifespan of males than females, and that the extent of this effect is dependent on the relative availability of food resources (Bedhomme *et al.* 2004, Sharmila Bharathi *et al.* 2007).

Another study has shown that an increase in the sexual activity of a male fly is correlated with a decrease in the ability to fight off infection, again indicating a fitness cost of the immune activation (McKean and Nunney 2001). Males housed with a greater number of virgin females were less able to clear *E. coli* infections than were males exposed to fewer females. The authors suggest that this decrease in the ability to fight off an infection is a result of the flies' spending energy on mating instead of the immune response, consistent with the hypothesis that there is a trade-off between immune activation and fly fitness (McKean and Nunney 2001). This effect is not limited to bacterial infections. Fly larvae that successfully survive infection with the parasitoid wasp *Acyrthosiphon pisum* Harris exhibit a reduction size and fecundity as adults, as well as an increased susceptibility to parasitoids during the pupal stage (Kraaijeveld *et al.* 2002). Infection of flies with various pathogens has therefore been observed to lead to a decrease in the adult flies' lifespan and ability to reproduce, and the authors cite immune activation as the reason for these fitness costs (Kraaijeveld *et al.* 2002). If these observed costs in fitness are exclusively the result of immune activation, and not other influences that the pathogen may exert on the fly, the use of immune activation to limit insect infection will be difficult or impossible, since these high fitness costs would preclude refractory flies' invasion and maintenance in nature.

However, these studies largely depend on (1) infection of the fly with various pathogens to initiate an immune response and (2) an attribution of any observed fitness effects to the immune deployment alone, ignoring the fact that the infectious agent may have a significant impact on biological processes of the host other than immune activity. In fact, the injection of high numbers of bacteria directly into the haemolymph of a fly, and any subsequent replication of these bacteria, is certain to have effects on the fly that far exceed simple immune induction. The bacteria will, for example, utilize fly resources for their own replication and may produce virulence factors that directly alter fly fitness, irrespective of immune activation. Also, this model of immune deployment leads to long-term, if not lifelong, immune activation, while transgenic insects can be designed to have only transient activation of an immune defence mechanism in a specific tissue compartment. To this end, another study investigated the effects of short-term immune activation mediated by the NF-κB transcription factor, controlled by the IMD pathway, using an inducible system in transgenic flies (Libert *et al.* 2006). In this system, long-term immune activation by constitutive over-expression of peptidoglycan recognition protein (PGRP-LE) in the fat body, that is known to activate the IMD pathway, led to a reduction in lifespan and a generally high fitness cost, as expected. However, when the IMD pathway was activated for only a few days at a time by PGRP-LE under the GeneSwitch-inducible GAL4 system and feeding the flies on food supplemented with mifepristone (RU486), the lifespan and other fitness parameters was not reduced and the flies behaved normally, as assessed by measuring their heat tolerance, geotaxis and reproductive ability. Also, contrary to the previous infection-based assays of *Drosophila* fitness, flies with transiently activated immune responses survived significantly longer after infection with *Pseudomonas aeruginosa* Migula than their conspecifics lacking immune up-regulation (Libert *et al.* 2006). This result indicates that much of the negative fitness effect observed in the previously mentioned studies was likely due to the presence of the bacteria and a long-term immune activation, and not a transient transgenic immune response. If there were a cost to simply activating the immune system on a short time scale, flies with the inducible PGRP-LE would display a fitness cost similar to that of flies with constitutive expression, and they would not exhibit an increased lifespan after infection with various bacteria. Therefore, while immune activation certainly can lead to manifold negative fitness effects, there is also evidence that tissue-specific, short-term immune deployment is not detrimental to the insect's lifespan and may lead to a fitness advantage under certain conditions of infection.

Impact of immune response and transgene expression on mosquito fitness

While most research into the evolutionary costs of increased immune deployment has been performed in *Drosophila*, the results are only as relevant as the model organism employed: while *Drosophila* serves as a valuable genetic model, there are many differences between flies and important disease vectors. The fact that mosquitoes and other vectors of human disease are haematophagous adds a new dimension of complexity to their fitness, given that a blood meal may provide sufficient nutrients to make up for any reallocation of resources for the purpose of producing immune effectors. Conversely, the acquisition and digestion of a blood meal both require significant energy expenditure, given the challenge of finding a suitable host, breaking down the blood proteins to useable units and dealing with the many toxic compounds produced during blood digestion, such as heme and reactive oxygen species (Zhou *et al.* 2007). This necessary energy usage may only compound any energy shortages caused by immune deployment, again leading to a complex and somewhat unpredictable set of interactions that will affect the fitness of mosquitoes that are found or created to be refractory to disease transmission.

There is, however, evidence of a potential effect of immune activation on mosquito fitness, similar to that observed in *Drosophila*. A number of studies have indicated that infection of *Anopheles* mosquitoes with *Plasmodium* parasites reduces the lifespan and reproductive output of the mosquitoes (Anderson *et al.* 2000, Hogg and Hurd 1995). *Ae. aegypti* adults selected to be resistant to *P. gallinaceum* are significantly smaller, lay fewer eggs, and have shorter lifespans than susceptible conspecifics (Yan *et al.* 1997). These differences are not unexpected, given that similar results have been observed in *Drosophila* and because there is significant conservation between the *Drosophila* and mosquito immune systems (Christophides *et al.* 2002). Conversely, male *An. gambiae* from a line selected for increased melanotic encapsulation of *Plasmodium yoelii* show an increase in fecundity, as measured by the number of offspring born to their mates (Voordouw *et al.* 2008). Similarly, Dong *et al.* (2011) observed that transgenic mosquitoes that over-express the Rel2 transgene upon induction of the carboxypeptidase promoter following a bloodmeal have no reduction in longevity when fed only on sugar. Mosquitoes provided with a naïve blood meal likewise showed no reduction in longevity, while mosquitoes fed upon *P. falciparum* infected blood exhibited a minor reduction in lifespan, as well as a modest reduction in the number of eggs laid (Dong *et al.* 2011). These studies, taken together, show that an increased immune activity in mosquitoes may have disparate fitness effects, depending on the host-pathogen system, and that not all effects are negative. Also, careful measurement of the fitness costs imposed on the mosquito by both infection with *P. yoelii* and resistance showed that increased melanotic encapsulation of parasites has the same cost; both in terms of lifespan reduction and egg hatch rate (Hurd *et al.* 2005). Thus, a moderate fitness cost resulting from increased immune activation may be acceptable, since it will simply offset the fitness cost of infection.

In addition to any fitness effects caused by immune activation in mosquitoes, there may be effects related to genetic manipulation itself (Marrelli *et al.* 2006). Transgenesis allows the introduction of novel genes that can lead to refractoriness and also allows transient immune activation instead of constitutive up-regulation, which can limit any negative effects of immune over-expression, as discussed above. However, the creation of transgenic mosquitoes can carry with it an inherent cost to the transformed insect. Genetically modified mosquitoes made to constitutively express a green fluorescent protein after insertion with the *piggybac* transposable element have a competitive disadvantage when compared to both wild-type and inbred, but not transgenic, mosquitoes when reared together (Koenraadt *et al.* 2010). The negative effects of transgenesis were only compounded when limited food resources are provided and the adult transgenic mosquitoes have fewer energy reserves available. Thus, exogenous gene expression utilizes energy that would otherwise be used for development (Koenraadt *et al.* 2010). In a separate study, Amenya *et al.* (2010) created mosquitoes expressing an enhanced cyan fluorescent protein inserted into an *attP* docking site and saw no decrease in transgenic mosquito lifespan or fecundity (Amenya *et al.* 2010). Use of an *attP* docking site takes advantage of the site-specific integration of the φC31 integrase to insert transgenes into a known chromosomal location (Nimmo *et al.* 2006). By doing so, different transgenic lines can be created with the gene of interest inserted into a position with known fitness effect, allowing both minimization of negative fitness effects and the measurement and comparison of the effects of different transgenes on fitness independent of effects due directly to insertion. Li *et al.* (2008) observed no measurable effect on the adult survivorship, egg hatch rate or larval-to-pupal viability in *An. stephensi* mosquitoes that express the exogenous peptide SM1 under the carboxypeptidase promoter. However, during the same study, when the authors kept cages containing both transgenic and wild-type mosquitoes for multiple generations, they noticed that the frequency of genetically modified mosquitoes decreased over time. They attributed this effect to a lower reproductive capability of the transgenic mosquitoes or a negative consequence of the insertional mutagenesis, and not the expression of the transgene (Li *et al.* 2008). The same

group also observed that the transgenic mosquito line expressing SM1 has a fitness advantage over wild-type conspecifics upon infection with *P. berghei* (Marelli *et al.* 2007). This effect was seen not only in the form of a higher fecundity and longer lifespan in one generation, but also in the gradual replacement of wild-type mosquitoes by the genetically modified mosquitoes over multiple generations when fed on *P. berghei*-infected mice, but not when fed on uninfected mice (Marrelli *et al.* 2007). Taken together, these studies show that any effects of transgenesis on the mosquitoes will depend on the environment in which the mosquitoes live. These types of effects can be avoided by selection of the most fit transgenic lines after many have been created; however, if the effects are only slight reductions in lifespan or fecundity, they may not be noticed during the selection process. New methods of transgenesis that allow site-specific integration of the transgenes have recently been developed, allowing the selection of the insertion location and minimization of gene disruption (Amenya *et al.* 2010, Labbe *et al.* 2010, Meredith *et al.* 2011). However, it is more likely that any transgene introduced into the mosquito will lead to effects that reflect a reallocation of resources to producing the transgene, as previously described for immune activation. Also, an inserted gene may have more widely ranging effects than initially predicted, potentially leading to greater resource use or significant changes in gene expression. For instance, if an inducible transgene that affects both immune and developmental functions is introduced, the result may be a differential expression of numerous genes beyond the initially targeted immune genes, and therefore widespread effects on the mosquito and a greater fitness cost. Such effects, however, can be minimized by carefully selecting the gene to be introduced, expressing it in a highly tissue- and stage-specific manner and creating multiple transgenic lines, then monitoring and selecting the line with the least observable effect on lifespan, fecundity and other fitness measures before the insects are released. When multiple transgenic lines with the same transgene are created through random integration, both the expression of the transgene and the effects of integration on other genes can vary greatly. This variability is the result of position effects, i.e. variability in the expression of a gene that is a consequence of its location on the chromosome, and therefore its relative proximity to other genes or regulatory elements that act on all genes within their reach (reviewed in Wilson *et al.* 1990). Furthermore, the effects of the transgene on neighbouring genes will vary greatly depending on its final location: whether it has interrupted a gene or a regulatory sequence, or the interactions between the two. Thanks to our extensive knowledge of these effects, careful design of transgene constructs and selection of transgenic strains can minimize these effects of transgenesis.

It is also important to note that a small decrease in the fitness of a vector as a result of increased immune deployment or transgenesis would not preclude using this system as a vector-borne disease control technique. As discussed above, the vectorial capacity of an insect vector depends on numerous factors, including both the vector competence and daily survivorship of the vector. Activation of a specific arm of the innate immune system so as to reduce the ability of the mosquito to transmit a pathogen is, in effect, decreasing the vector competence of the mosquito and producing a related decrease in vectorial capacity. However, a decrease in daily survivorship, such as one caused by a fitness cost associated with gene expression, will also decrease vectorial capacity. Likewise, a decrease in survivorship or in the number of eggs produced by each generation will lower the mosquito density relative to human hosts, yet another factor that can lead to an overall reduction in vectorial capacity. Overall, a slight compromise in mosquito fitness leading to decreased fecundity and lifespan can lead to a large decrease in vectorial capacity, especially if combined with an additional reduction in vector competence. However, any decreases in fitness must be limited in scope so that they do not prohibit the genetically-modified insect from invading the natural population and maintaining a normal population; otherwise, the modified mosquitoes will never reach high enough numbers to be a viable tool for vector control. Use of

strong gene drivers, such as homing endonucleases or *medea* elements, should be able to mitigate some of the fitness cost and still drive the genes of interest into the natural population (Walker and Moreira 2011, Windbichler *et al.* 2007, reviewed in Cirimotich *et al.* 2011). This goal can be met by increasing the probability that a gene will be spread to the mosquitoes' offspring or by giving mosquitoes bearing the gene driver a significant fitness advantage over those that lack the gene driver, such as by killing offspring that lack the driver. As long as the transgene being introduced into the mosquito population is linked to the gene driver, it will be carried with the driver, and the genetically modified mosquitoes will replace the wild-type population to a transgenic population.

Conclusions

Mounting a proper immune response when infected with a pathogen is integral to the survival of any organism, including insects. However, an overactive immune system can also cause damage to the host through inflammation and over-utilization of limited resources. Therefore, creating transgenic mosquitoes that over-express immune genes to combat vector-borne disease in the absence of a powerful genetic drive system may be difficult to implement in the real world. Multiple studies in *Drosophila*, as well as more limited evidence from other insect systems, have shown that long-term activation of the immune response brought about by infection with bacteria can lead to a decrease in lifespan and other common fitness measures. However, studies based on transient activation of the immune system have exhibited no such fitness cost, indicating that short-term expression of immune effectors may not have a negative effect on insect fitness and indicating that this approach may represent a viable technique for vector-borne disease control. Combining a gene for refractoriness with a gene drive mechanism, such as a homing endonuclease or the intracellular bacterium *Wolbachia*, will drive the refractoriness into the natural vector population, even if there is a small fitness cost (Walker and Moreira 2011, Windbichler *et al.* 2007). Also, a modest fitness cost attributed to life-span or reduction in the ability to obtain a second blood meal will decrease the vectorial capacity of the vector further than will a simple decrease in vector competence alone, by simultaneously lowering the vector density and limiting the daily survival rate. Therefore, while there may be some fitness cost associated with over-expression of immune genes, these negative effects can be limited by implementing only transient expression of the transgene in a tissue- and stage-specific manner, and connecting the gene for refractoriness to a strong gene driver, thereby generating mosquitoes over-expressing immune genes that represent viable agents for disease control.

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3. Plant-sugar feeding and vectorial capacity

Chris M. Stone and Woodbridge A. Foster

Abstract

Sugar feeding is a common behaviour of male and female mosquitoes, sand flies, and other Dipteran vectors. In some species it is essential to one or both sexes; in others it is facultative. Even among females of anthropophilic species that are predisposed to a diet of frequent blood meals sugar is often taken, depending on internal state and opportunity. This opportunism is expressed as an increased likelihood of feeding on nectar when access to blood and oviposition sites is limited. Newly emerged *Anopheles gambiae* females sometimes show a preference for sugar before mating even when blood hosts are available, likely depending both on the strength of plant and animal kairomones and on the attractive qualities of each. Incorporation of sugar in the diet by mosquitoes affects certain components of their vectorial capacity. Environmental conditions, such as bed net coverage and abundance of nectar sources, will affect the extent to which mosquitoes feed on sugar. If the effect of sugar on vectorial capacity is significant, these conditions will impact transmission rates of vector-borne diseases and should be included in epidemiological models. Vectorial capacity is pulled in opposing directions by sugar feeding, through its effect on the two most important components, survival and biting rate. Survival of females feeding on sugar and blood is greater than that of females restricted to a blood-only diet, according to the vast majority of studies, whereas biting rates usually are depressed when sugar is available, but field evidence is scarce. Vector density results from survival and fecundity. Most studies on vectors suggest that although fecundity per gonotrophic cycle is enhanced by sugar feeding, long-term reproductive fitness in anthropophilic species is slightly depressed. Vector competence appears to be negatively affected by sugar feeding. In certain cases plant nectar contains factors that inhibit development of the parasite in the vector. More common may be positive effects on the vector's immune response, but this appears to depend heavily on the host-parasite system, condition of the vector, and possibly genotype-by-environment interactions. Estimating the combined effect of these factors at different levels of sugar intake remains difficult at this point, but an overall impression is that vectorial capacity is somewhat decreased in environments where sugar is readily accessed. Sugar feeding behaviour can be exploited for control, the most promising methods employing sugar solutions combined with attractants and oral insecticides for direct control and attractive phytochemicals for surveillance. Main questions facing both approaches are their suitability in verdant areas where attractants will compete with a diverse flora. For females of anthropophilic species in settings with abundant blood hosts, the question may be whether populations can be effectively suppressed by targeting male mosquitoes.

Keywords: nectar feeding, vectorial capacity, fitness, novel control methods

Introduction

Overviews of insect-vector sugar feeding (Downes 1958, Foster 1995, Yuval 1992) identified important gaps in our understanding of the process and its implications. One particularly important gap was the influence of available plant sugar on vector populations: whether it is a limiting resource, so that its restriction can affect reproductive success and survival, and therefore the sustained density of adults, i.e. the carrying capacity of the environment. Another gap, equally important, was the effect of plant sugar on pathogen transmission, including how the availability of sugar can affect vectorial capacity by altering vector competence or by changing biting

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frequency and survival. Because density of adults also contributes to vectorial capacity, these two gaps in our knowledge are part of the same question for disease ecologists: is plant-sugar feeding by vectors a critical component of pathogen transmission?

Investigations of sugar feeding vector biology, both in the laboratory and in the field, made since those comprehensive reviews, are beginning to provide details that can fill these gaps. This review will focus mostly on newly published work and on aspects of the plant-vector topic not previously discussed. For the earlier literature supporting conclusions and generalizations about mosquito sugar feeding, summarized in the present review but not fully referenced, the reader is referred to Foster (1995). For malaria transmission in particular, the possibility that sugar feeding by *Anopheles* mosquitoes may be an essential component of the epidemiological process is gaining wide recognition (Ferguson *et al.* 2010). Its importance for leishmaniasis transmission by phlebotomine sand flies in desert regions also is strongly supported. Experiments in both disease systems are providing direct evidence for plant-sugar's pivotal role in vector biology and offering ways to manipulate the connection between plant and vector to weaken or eliminate pathogen transmission, either by itself or as a valuable component of integrated control (Beier *et al.* 2008; Shaukat *et al.* 2010).

Taxa involved and evidence

Taxa covered

Most blood-feeding Diptera also ingest plant sugar. For this reason, it is accurate to say that they have two types of hosts: vertebrate animals and vascular plants. These dipterans include the blood-feeding species of the families Culicidae, Ceratopogonidae, Simuliidae, Psychodidae, Tabanidae, Rhagionidae, and some blood-feeding Muscidae. Notable exceptions are the tsetse flies (Glossinidae) and the ectoparasitic pupiparous dipterans (Hippoboscidae, Streblidae, and Nycteribiidae). For a few poorly known haematophagous flies, e.g. Corethrellidae and Carnidae, the role of sugar feeding has not yet been established. No other haematophagous insects are known to take plant sugar. These blood feeders include the Siphonaptera, among Hemiptera the triatomine Reduviidae, the Cimicidae, and the Polyctenidae, and among Phthiraptera the anoplurans, amblycerans, and rhynchophthirines. This also appears to be true of all haematophagous Acari. So far, the great majority of sugar feeding investigations have targeted mosquitoes (Culicidae) and the phlebotomine sand flies (Psychodidae). However, recent important plant-related studies include horse flies and deer flies (Tabanidae), black flies (Simuliidae), biting midges (Ceratopogonidae), and stable flies (Muscidae).

Plant food sources and composition

The sugar feeding haematophagous Diptera obtain their sugar from a variety of plant sources, most commonly floral and extrafloral nectar and honeydew; the latter is plant-derived but homopteran-produced. Other sources are damaged or decaying fruit and seeping sap from plant wounds. Typically, these flies direct the ingested vertebrate blood straight to the midgut, where most digestion and all absorption occurs. They shunt all but the smallest sugar meals to the foregut diverticula, blind sacs where sugar is stored prior to being doled, a little at a time, into the midgut for digestion and absorption. Most of a sugar meal is stored in the large ventral diverticulum, the crop. Although the sugars sucrose, fructose, and glucose are the primary constituents of nectar, minor sugars also occur, and various oligosaccharides are common in honeydews. Glycosidases in mosquito and sand fly saliva (Jacobson and Schlein 2001, James and Rossignol 1991, Marinotti

and James 1990) and midgut (Billingsley and Hecker 1991, Jacobson and Schlein 2001, Souza-Neto *et al.* 2007) cleave sucrose into its constituent hexoses: fructose and glucose. In addition, plant-sugar meals usually contain amino acids and are considered to be part of the flower-pollinator and extrafloral gland-mutualist syndromes (Shuel 1992). The amino acids by themselves are insufficient to stimulate or support mosquito egg development, but they do appear to promote survival (Eischen and Foster 1983, Jones *et al.* 1985, Vrzal *et al.* 2010) and also may serve as a flight substrate (Scaraffia and Wells 2003). Many other nectar constituents have been found, some presumably nutritional, others distasteful or toxic.

Tissue piercing

Healthy plant tissue is sometimes pierced, and sugars and other nutrients are then extracted from phloem sap or tissue fluids. Tissue feeding has been examined most extensively and intensively in desert sand flies, where it appears to be essential to their survival. In mosquitoes, this phenomenon has been reported many times in the literature, yet it has not been explored intensively, and its significance for them globally remains unclear. The most recent evidence for mosquito and sand fly tissue feeding comes from studies in Israel, where calcofluor-stained cellulose particles have been detected in the midgut (Junnila *et al.* 2010, Müller and Schlein 2005, Müller *et al.* 2010b, Schlein and Jacobson 1999, Schlein and Müller 1995), and chloroplast DNA has been identified (Junnila *et al.* 2010). Amylase activity has been detected in many haematophagous flies (reviewed by Gooding 1975), including sand flies and mosquitoes, and amylase gene expression has been explored in mosquitoes (Grossman *et al.* 1997). In phlebotomine sand flies it is used to digest starch granules obtained during plant tissue feeding (Jacobson and Schlein 2001, Jacobson *et al.* 2001, Ribeiro *et al.* 2000). The fluid ingested by sand flies during plant piercing is transferred directly to the midgut (i.e. in the 'blood-feeding mode'), rather than being shunted into the crop (Schlein and Warburg 1986). The presence of amylase in mosquitoes and other haematophagous Diptera suggests that starch, derived from tissue feeding, is part of the diet. In both sand flies and mosquitoes it is reported to be particularly common during seasons and localities when and where sugar sources are rare and plants are under heat and water stress (e.g. Müller *et al.* 2010c, Schlein and Jacobson 1999, Schlein and Müller 1995). Tissue piercing may provide water as well as sugar, but it also affects *Leishmania* infections in sand flies (Schlein and Jacobson 2001) (see below).

Methods for evaluating plant feeding

The evidence for sugar feeding in the field, and much of what we know about it, comes either from direct observations of insects on plants or from chemical tests of gut contents. Observations of vector behaviour on plants are sometimes difficult to interpret, because landing, aggregating, and even probing do not necessarily result in ingestion of sugars. Furthermore, the failure to observe plant-feeding behaviour, and therefore deduce its absence, is notoriously misleading. Sugar feeding often occurs rapidly and over broad periods of the insect's activity period, spread over broad sweeps of a landscape that supports a vector's host plants. This is unlike blood-feeding behaviour, which tends to be concentrated on relatively scarce hosts and consequently is more obvious to the human observer.

Chemical tests are less susceptible to sampling biases than direct observations. The one that revolutionized sugar feeding studies of vectors is Van Handel's cold-anthrone test (Van Handel 1967, 1972) for fructose, a plant sugar not synthesized *de novo* within the insect. Other methods for detecting fructose have been developed that are reported to give greatly increased sensitivity (Nunes *et al.* 2008, Somani *et al.* 1987). Simple chromatographic methods also have proved

satisfactory for detecting undigested sugar meals, provided that they distinguish between plant-derived and metabolically generated sugars (Laarman 1968, Magnarelli and Anderson 1977, Magnarelli 1978, 1979, 1980, Nayar 1978, Watanabe *et al.* 1973). Particularly useful have been thin-layer and gas chromatography that identify sugars distinctive to honeydew, as opposed to floral and extrafloral nectars (Burgin and Hunter 1997a,b,c, Burkett *et al.* 1998, 1999, Hunter and Ossowski 1999, Janzen and Hunter 1998), or that can help determine the likely plant-host species by their sugar ratios (Hamilton and El Naiem 2000, Manda 2007a). Finally, evidence for the penetration of undamaged plant tissue, such as leaves and stems, can be deduced from the presence of dyes and cellulose (e.g. Müller *et al.* 2010b, Schlein and Jacobson 1999, Schlein and Müller 1995), and the plant-host species can be identified from chloroplast DNA (Junnila *et al.* 2010). The principal problem we confront with chemical tests of gut contents is that sugars and other materials disappear either gradually or rapidly, depending on the amount consumed and rates of digestion and egestion in each species according to temperature and the individual's physiological status. Many negatives will be recorded, even among species that plant-feed at frequent intervals. So the tests provide only an approximation of the proportion of vectors that have fed on plants during a particular period of time.

An underutilized low-bias approach to determine the identity of plant hosts is the identification of pollen grains on the body or mouthparts, or in the gut. Pollen has been used effectively as an indicator of specific plant visits by the tabanids *Tabanus* and *Chrysops* (Magnarelli 1979) and the stable fly *Stomoxys* (Jarzen and Hogsette 2008, Tseng *et al.* 1983). Pollen also has been found on a wide variety of mosquito species, some of which are implicated as pollinators. Pollen information is limited by the uncertainty of the time that the pollen was acquired. In addition, some pollen grains are too large to be incorporated in pollen-contaminated nectar meals of insects with narrow food tubes or are held in pollen-transfer devices that do not adhere to the bodies of small nectar thieves. Also, pollen will not account for a vector's visits to non-floral sugar sources, and cross-contamination of pollen between plant species conceivably may lead to false conclusions.

General features of plant feeding behaviour

Autogeny and diapause

A good baseline of knowledge about sugar feeding behaviour in mosquitoes exists, derived from a variety of lab and field studies. In the case of females of autogenous species, a sugar meal often is necessary for the development of the first batch of eggs (O'Meara 1985, 1987). Even among anautogenous mosquitoes there are species that rarely or never seek blood until they take at least one sugar meal (Briegel *et al.* 2001, Hancock and Foster 1997, 2000, Renshaw *et al.* 1994, 1995). Sugar feeding frequency may diminish in females of some anautogenous species, once insemination is achieved and blood feeding commences, whereas in males it remains constant throughout life. On the other hand, where winters are severe, females that have entered a state of adult diapause prior to overwintering either do not take blood at all (*Culex*) or take non-ovigenic blood meals close to their hibernacula (

Anopheles). However, *Culex* females entering diapause up-regulate genes for fatty acid synthase (Robich and Denlinger 2005, Sim and Denlinger 2009), and indirect evidence indicates that they feed on sugar frequently prior to entering hibernacula (Bowen 1992a, Jaenson and Ameneshewa 1991). During this period they accumulate large reserves of fat before foraging becomes impossible. Where winters are milder, some sugar feeding may occur among diapausing *Culex* populations

throughout the winter (e.g. Reisen *et al.* 1986), perhaps explaining the sporadic expression of the fatty acid synthase gene throughout simulated hibernation.

Food utilization

Ingestion of sugar is directly correlated with flight range, and sugar can be consumed directly as a flight-energy substrate. Alternatively, it may be converted to glycogen for storage in the insect's fat body and flight muscles. Although previously thought to be used primarily for survival, stored lipid derived from either blood or sugar also can serve as a flight substrate in *Anopheles gambiae* Giles, which has an exceptional ability to mobilize lipid during long flights (Kaufmann and Briegel 2004), likely involving adipokinetic hormone. There is evidence that even amino acids derived directly from the blood meal may be used in flight in *Aedes aegypti* Linneaus (Scaraffia and Wells 2003).

Timing and frequency

The first adult food of both sexes of anautogenous species is likely to be plant sugar, and both sexes continue to take sugar throughout their reproductive lives. Sugar feeding by females appears to be least likely to occur when they are digesting a blood meal and most often when gravid or prior to the next blood feeding. But there are many exceptions to this, depending both on species and circumstances. Sugar feeding has a characteristic time, or times, in the diel activity cycle. Recent studies have started to elucidate the molecular basis of this rhythmic behaviour (e.g. Rund *et al.* 2011). Cycles of sugar feeding often share a general activity rhythm with other behaviours, so that the phases of different behaviours are the same or nearly so. A field study of *An. gambiae* by Müller *et al.* (2010b) demonstrated that the times of sugar seeking and blood seeking, though to some extent overlapping, occurred in distinctly different parts of the diel cycle: attraction to sugar baits occurred mainly early in the night, with a second peak shortly before dawn, whereas attraction to blood-host odour occurred mainly in the second half of the night, in accord with landing or biting rates of other studies of this species.

Average sugar feeding frequency is difficult to infer from field data, because of strong temperature fluctuations and narrow periods when feeding occurs. Rough estimates are based on the time for all individuals to digest naturally acquired nectar meals completely and on the fraction of resting individuals that contain a meal still in some stage of digestion. By extrapolation, the time spent without sugar is calculated, and the total time with and without sugar provides the sugar feeding interval. Typical values indicate that males in the field may take sugar every 1-2 days, whereas mature females of anthropophilic species may take it as infrequently as every 6-9 days. These species are less dependent on sugar for successful reproduction, flight, and extended life in the laboratory (Fernandes and Briegel 2005, Harrington *et al.* 2001, Kaufmann and Briegel 2004), and they less often contain undigested sugar meals in the field (as explained below). Females of typical zoophilic species, by contrast, often contain undigested sugar and probably feed on sugar at least as frequently as on blood, i.e. every 3-4 days. Without sugar, they can die rapidly, despite frequent access to animal or even human blood (Fernandes and Briegel 2005, Nayar and Sauerman 1975, Wittie 2003).

Limited and limiting availability in the field

In laboratory cages, sugar availability clearly alters survival and reproduction. Evidence from the field is much harder to come by, primarily because of difficulties in measuring the accessibility of sugar in nature. Field samples support the general notion that more sugar feeding occurs

when more sugar sources, or just more plants, are available (Martinez-Ibarra *et al.* 1997, Müller *et al.* 2010d). These studies suggest the hypothesis that plant sugar is a limited, and potentially fitness-limiting, resource. The underlying assumption is that up to some unknown point, it is advantageous for vectors to take more sugar if they can find it. In several studies (e.g. Gadawski and Smith 1992, Hocking 1953, 1968), vector population density was low if fewer preferred nectar sources were available, or declined seasonally when the sugar sources declined. Some of the best evidence for this effect comes from the few studies of sand flies and mosquitoes in isolated areas in which a suspected plant host either was marked with a sugar-baited dye or was sprayed with insecticidal bait (Müller and Schlein 2006, Schlein and Müller 2008). Another approach was to compare the reproductive performance of cohorts in mesocosms with and without sugar sources (Stone *et al.* 2009), or to measure the density and survival of populations of vectors in isolated areas that appear to differ only in the availability of certain preferred plant hosts (Gu *et al.* 2011). In the last case, a drastic difference in survivorship and biting frequency has been attributed to the presence or absence of a single species of host plant (See more in the Section 'Vectorial capacity').

Plant-host preference

Mosquitoes and other vectors collected while standing, crawling, or probing on a variety of plants in bloom appear to show preferential attraction to certain plant species, because they occur disproportionately on those plants. This apparent degree of host specificity is reinforced by the many anecdotal observations that link an insect to only one or a few plant species. Another source of evidence for plant-host selectivity is the ability of vectors to obtain fluids, secretions, or nutrients from only a small subgroup of the species in a plant community. For example, Abdel-Malek and Baldwin (1961) were the first to suggest selective removal of sugar-bearing plants as a means of control (see Section 'Selective removal of plants'). They found that *Ae. aegypti* and three indigenous Canadian mosquitoes fed on only three of 24 native plant species offered to them. A study in a natural setting in Egypt likewise revealed that *Anopheles sergentii* Evans males fed on a very select number of plants, and the presence of those plants predicted the presence of larvae in nearby pools. Furthermore, field collections of males were successful on these plants, whereas very few *An. sergentii* were collected from other plants (Abdel-Malek 1964). Similar laboratory experiments demonstrating differences in mosquito and sand fly ingestion and survival on various plant species have been conducted by Patterson *et al.* (1969), Schlein and Warburg (1986), Alexander and Usma (1994), Gary and Foster (2004), Impoinvil *et al.* (2004) and Manda *et al.* (2007b).

Even investigators who take into account the relative availability of all possible host plants can misinterpet insect aggregation – possibly the result of behavioural arrest – as attraction. Recent sand fly and mosquito experiments have eliminated some of this bias and confirm that vectors have plant preferences. These experiments have used plant-baited or plant-associated traps and resting sites (Schlein and Yuval 1987, Müller and Schlein 2004, Gouagna *et al.* 2010), selective dye-marking or insecticide treatment of plants in the field (Schlein and Jacobson 1994, Schlein and Müller 1995, 2008, Müller *et al.* 2010b, Müller *et al.* 2011), radioactive tagging of plants (Abdel-Malek and Baldwin 1961, Abdel-Malek 1964, Patterson *et al* 1969), and wind-tunnel olfactometers (Gouagna *et al.* 2010). The existence of strong differences in attraction of *An. sergentii* to specific plants was convincingly demonstrated by miniature CDC-light traps baited with branches of potential plant hosts (Müller and Schlein 2006). Müller *et al.* (2010a) also found evidence for differences in attraction of *An. gambiae* to fruits and flowering plants in Mali using wire-mesh glue traps surrounding 26 plant species and 26 kinds of fruits and seedpods. Flowering plants

were considerably more attractive than fruit, and over all, nine out of the 26 plants tested were considered attractive, with only minor differences between males and females.

A completely unbiased form of evidence for selective plant-feeding (i.e. the host-utilization rate) in nature must come from the guts of random samples of vectors collected in the field. These kinds of data, when combined with knowledge of the proportions of different plant species available to the vector, provide a measure of plant preference (i.e. the forage ratio or feeding index). Rigorous studies of this kind have yet to be conducted. However, chromatographic profiles of sugars in *Phlebotomus orientalis* Parrot sand flies in the Sudan demonstrated the relative importance of fruit and honeydew (Hamilton and El Naiem 2000) and in several species of mosquitoes (Burkett *et al.* 1999), black flies (Burgin and Hunter 1997a,b,c), deer flies and horse flies (Hunter and Ossowski 1999, Janzen and Hunter 1998). Chloroplast nucleotide sequences showed that during the dry season *An. sergentii* mosquitoes mainly tissue-fed on three succulent species that formed less than 1% of the vegetation (Junnila *et al.* 2010).

Obligatory vs. facultative nature of sugar feeding

Anthropophilic and generalist species

Among mosquito species there appears to be a continuum of female reliance upon sugar feeding. At one extreme, *Toxorhynchites* females feed exclusively on plant sugars, relying for egg production on the protein reserves from the larval stage. At the other extreme, where human blood is readily available, certain species have been reported to contain plant sugar infrequently or rarely (Beier 1996, Costero *et al.* 1998, Edman *et al.* 1992, Gillies 1968, Spencer *et al.* 2005). Females of these species may rely on blood entirely, often feeding multiple times per gonotrophic cycle (e.g. Beier 1996, Braks *et al.* 2006, Edman *et al.* 1992, Foster and Eischen 1987) or having overlapping gonotrophic cycles (Briegel and Hörler 1993). Nonetheless, such females readily feed on sugar in the laboratory, and a modest proportion can be found to contain sugar in the field.

Species for which facultative sugar feeding has been proposed are *An. gambiae* and *Ae. aegypti*. They occupy a specialized niche and share a number of characteristics, in particular anthropophily and endophily, that predispose them to a diet limited to blood. Due to their tendency to rest indoors after feeding on blood, a female's energy requirement for flight is limited to that needed for seeking hosts, mates and oviposition sites; the energy required to locate any of these, near domiciles, probably is small or negligible. The mating behaviour of *Ae. aegypti* is notable in that swarming and mating occur around the blood host, instead of above inanimate swarm markers. Yuval (2006) proposed that this behaviour may be an adaptation to dispersed and non-synchronous emergence of adults. Alternatively, it may be simply a result of intense intrasexual selection. *An. gambiae* do mate in conventional swarms, but the flight energy a female might expend to locate the swarm, mate, and return to a resting site, has not been examined. The discovery that mating in this species sometimes occurs indoors in West Africa (Dao *et al.* 2008) implies that in some areas the energetic costs of mating and host seeking likewise may be small and overlapping, as in *Ae. aegypti*.

The lower incidence of sugar feeding, and higher rate of multiple blood feeding per gonotrophic cycle is likely one of the reasons both *Ae. aegypti* and *An. gambiae* are such efficient vectors of human disease. These characteristics may have evolved in response to an oddity of human blood composition. One of the amino acids essential for vitellogenesis, isoleucine, is notably limited in human blood compared to that of other vertebrates (Briegel 1985, Dimond *et al.* 1956, Lea *et al.* 1958). Consequently, a smaller proportion (up to 30% less) of each blood meal can be used for

gametic functions, allowing for a greater investment of blood-meal carbon to somatic functions. Kaufmann and Briegel (2004) provided an elegant example of this differing physiological reliance on sugar by comparing the flight distances of *An. gambiae* and *Anopheles atroparvus* Van Thiel when fed on sugar or blood. *An. gambiae* females mobilized their lipid reserves for flight and were capable of flying similar distances after being fed on sugar or on two blood meals. The more generalist blood feeder *An. atroparvus*, in contrast, did not use lipids to fuel its flight and achieved greater flight distances when fed sugar.

Mosquitoes feeding on blood hosts with higher isoleucine contents may thus be expected to have a higher reliance on sugar for the maintenance of energetic reserves. Whether prior sugar feeding by generalist mosquitoes, in turn, affects blood-host choice is not known. Even among anthropophilic species, under certain circumstances non-human animals may form larger proportions of their blood meals. Unclear at this point is whether feeding on non-human animals elevates their tendency to take sugar meals. This notion is supported by a comparison among experiments that have given markedly different survival and fecundity results, depending on whether the host was human, bird, or rodent. One example from a single study demonstrated that *Ae. aegypti* females, fed on human blood, had superior lifetime fecundity when sugar was absent; but when fed on mouse blood, fecundity was higher when sugar was present. Individual mouse blood meals, supplemented by sugar, generated by far the largest egg output (data courtesy of L. Harrington to W.A.F.). Yet, per mg, mouse blood alone was significantly less productive than human blood with sugar (Harrington *et al.* 2001). Lifetime survival showed a similar relationship except that there was no difference in survival on human blood, with or without sugar access. Studies on *Aedes albopictus* Skuse, which more often takes animal blood meals in nature, demonstrated that on human blood, females without sugar had moderately reduced survivorship (Braks *et al.* 2006). But on bird blood, females without sugar had drastically shortened lives (Xue *et al.* 2010).

Field evidence

The field evidence for a low incidence of plant-sugar feeding by anthropophilic species is not unequivocal. Of particular concern for the interpretation of fructose-positivity rates of field-collected females, in addition to those previously described, is the likelihood of collecting host-seeking or blood-fed females in indoor resting catches, potentially under-representing females in states that may be more inclined to sugar feed. Compounding these issues is the high variation reported in fructose rates between geographical areas and seasons, the main question being whether this variation is best ascribed to differences in plant community composition and abundance, or to the presence and availability of preferred blood-hosts.

Several field and laboratory studies provide insight into the facultative nature of sugar feeding of *Ae. aegypti* and *An. gambiae* and its variation across different habitats. For instance, wild *Ae. aegypti* were collected in a rural village in Thailand and tested for the presence of sugar during both the wet and the dry season (Edman *et al.* 1992). Seasonality did not affect sugar positivity, and only 3% of females were sugar positive, vs. 35% of males, demonstrating that sugar sources were at least available. In another study in Thailand seasonality did affect sugar intake of females (Spencer *et al.* 2005). Further evidence of limited sugar intake was obtained by collecting males and females inside houses in San Juan, Puerto Rico (Van Handel *et al.* 1994). Only 2% of the collected females contained fructose, and all females that were blood-fed or gravid were fructose negative. This could not be explained by the absence of sugar sources, because houses, patios and backyards typically contained a large number and variety of plants that might serve as nectar sources and 51% of males were fructose positive. The results from these field collections are in

stark contrast with sugar-positivity rates of females collected at a rural site (a tire dump) near Vero Beach, Florida. There, collected females contained no eggs or blood, and 74% contained fructose, while 63% of males did so (Van Handel *et al.* 1994). The authors attribute this striking difference to the difference in blood-host abundance between an urban centre with a dense human population and the rural site where humans are rare. A different field study did attribute the extent to which *Ae. aegypti* feed on plant nectar on the abundance of plants (Martinezz-Ibarra *et al.* 1997). More females in the outskirts of Huixtla, Chiapas, Mexico, were sugar-fed (21%) than in the midtown area (8%). There was no difference in number of inhabitants per house between these areas, but there was a significant difference in the number of flowering plants between the areas. Besides the difference in abundance of plants, sugar feeding may have been affected by the availability of specific plants, because bougainvillea and hibiscus occurred in 71% and 53% of the houses with sugar-positive mosquitoes, though it is not clear whether these plants were also more common in houses with sugar-positive mosquitoes than in houses without (sugar-positive) mosquitoes. Thus, there are conflicting reports in the literature about whether sugar feeding by this species is driven by absence of blood-hosts or presence of adequate sugar sources.

Field evidence for the use of sugar by *An. gambiae s.s.*, and the effects of environmental conditions on this behaviour, are scarcer than they are for *Ae. aegypti*. Most field evidence suggests that sugar feeding is rare, although a study by Laarman (1968) suggests that feeding on sugar is a normal component of *An. gambiae* behaviour. Muirhead-Thompson (1951) considered sugar to be an unnatural food source for this species, and Gillies (1968) found little evidence of sugar feeding in females collected indoors, based on the absence of fluid in their crops. McCrae (1989) suggested the habitats of *An. gambiae* were characterized by a paucity of sugar sources, but did observe *An. gambiae s.l.* feeding on the extra-floral nectaries of *Avena macrostachya* (personal communication to W.A.F.). Indoor-resting catches and indoor-biting catches in Kisian and Saradidi, Kenya, revealed 'surprisingly low' proportions of female *An. gambiae s.l.* and *An. funestus* Giles with detectable fructose (Beier 1996).

Laboratory studies on the blood/sugar choice

Field observations are not easy to interpret in terms of how likely a female is to feed on sugar at particular times in her life. A number of laboratory studies provide insight on this subject and suggest that while *An. gambiae* may indeed not be an obligate sugar feeder, sugar is a viable option for their first meal (Foster and Takken 2004), and is increasingly used later in life where blood hosts or oviposition sites are less accessible or more distant from one another (Gary and Foster 2006). Based on the finding that *An. gambiae* would feed on a human 24 hr after emergence, and that non-oogenic females were able to convert as much protein and lipid into maternal reserves as oogenic females transferred to egg yolk, Fernandes and Briegel (2005) suggested that this species may be opportunistic in terms of its feeding behaviour, i.e. both sugar and blood meals allow for rapid increases in reserve levels, and whichever is encountered first may be taken. This idea was supported by studies in mesocosms that showed that the pre-mating meal taken by this species favours sugar, but a proportion does feed on blood instead, and a greater proportion does so in the absence of sugar sources (Stone *et al.* 2011). Sugar-related and human-related volatiles thus clearly compete, and the determination of the initial meal choice may depend on both the strengths of the competing stimuli and their qualities.

In *Culex nigripalpus* Theobald, a mosquito that strongly prefers to feed on sugar before feeding on blood, this situational decision-making was demonstrated by Hancock and Foster (1997). In a wind tunnel choice test between sugar sources (honey) and blood (small birds), the response

to either food increased with increasing number of dishes of honey or the number of birds. A comparable study on *An. gambiae* in a mesocosm showed that blood-host presence (a human sleeping in the mesocosm throughout the night, or being available only for one hr per night) and female size, but not abundance of *Senna didymobotrya* (Fresen) Irwin and Barneby, affected the sugar/blood choice of 1-day old females, which strongly favoured blood in this case (Stone *et*

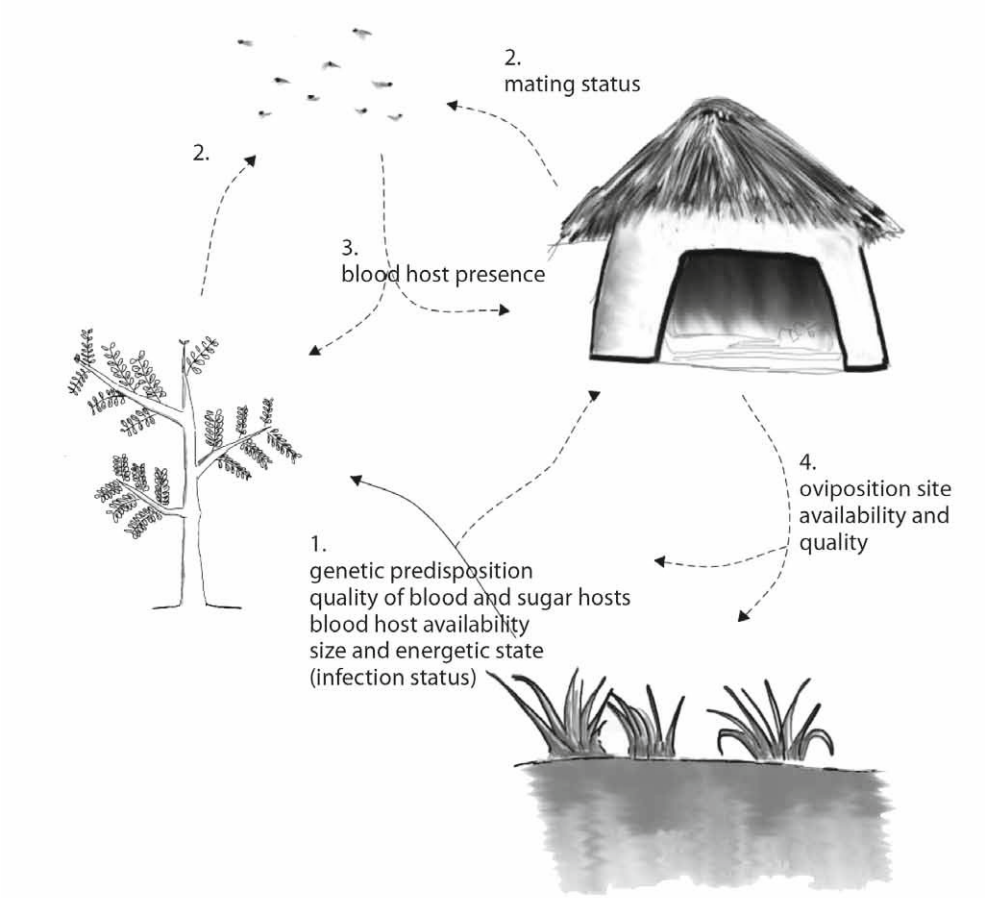

Figure 1. Life choice diagram for Anopheles gambiae *females, highlighting the behavioural components, the likely sequence in which they move through reproductive cycles, and factors informing the decisions between behaviours. (1) After emergence, or oviposition, females face a choice between seeking blood or nectar. (2) Depending on their mating status, females may seek out a male swarm. (3) Females will then seek a gonoinactive (= 'pre-gravid') or gonoactive blood meal, unless blood hosts are limited, in which case the likelihood of sugar feeding increases. (4) After a gonoactive blood meal, gravid females will oviposit, unless suitable sites are unavailable, in which case the likelihood of sugar feeding increases.*

al. 2012). This promotes the notion that females of this species are, rather than obligatory sugar

feeders, opportunistically inclined to use this resource at various points in their lives, depending on environmental conditions and resource accessibility and quality (Figure 1).

Sugar feeding by mosquitoes, according to optimal-foraging theory

Whether or not an animal should include in its diet an item with a particular energetic gain, probability of encounter, and handling time cost, is a question of classic foraging theory (Stephens and Krebs 1986). An assumption inherent to this theory is that animals will maximize their rate of energy input, which is then considered a proxy for fitness. As most animals have limited time-budgets and operate under constraints, and thus face trade-offs, using energy intake as a proxy for fitness is not always informative or free from error. An extension of classical foraging theory, dynamic state variable modelling (Clark and Mangel 2000, Mangel and Clark 1988), employs a fitness function that allows the maximization of a more relevant parameter. Typically this will be lifetime reproductive output. Theories based on these ideas could provide relevant insight into how the tendency to sugar-feed in certain mosquito species depends on environmental factors (e.g. resource availability). Additionally, mosquitoes in different behavioural states (age, mating status, body size, energetic reserve level, diapause status, etc.) differ in their tendencies toward taking sugar. To date, general theories applied to this have been developed (Roitberg and Friend 1992, Roitberg *et al.* 1994), and one study has investigated when to sugar-feed for the specific case of *An. gambiae* (Ma and Roitberg 2008). The main predictions from their model are that after emergence, a female is likely to take a sugar meal to increase her reserves before moving indoors to seek a blood host. Around houses, she seeks sugar only when energy levels become very low, and frequency of sugar feeding becomes negligible with increasing blood host availability. Following oviposition, she again commonly takes sugar – unless her energy reserves are high, in which case she returns indoors immediately. Longevity and fecundity both increase with increasing availability of sugar hosts outdoors and blood hosts indoors, whereas presence of peridomestic sugar hosts has negligible effects on these parameters. Several aspects that may be relevant to these predicted feeding choices have not yet been theoretically explored, such as the size or mating status of females. Another realm of questions that would benefit from model-driven hypotheses relate to how female mosquitoes infected with parasites may be expected to modify their feeding decisions (see Section 'Vector competence' below).

Vectorial capacity

Components of vectorial capacity

Vectorial capacity (C) is a simplified measure of a vector's power for pathogen propagation. It is expressed as the total number of new vertebrate cases of an infection that can arise directly from one original infection in a particular environmental setting, due to the insect or other carrier in question. It is a subset of the equations originally developed by Ross (1910) and MacDonald (1957) to provide a quantitative epidemiological description of pathogen transmission and spread among humans by anopheline mosquitoes, in terms of the reproduction rate of cases of malaria. Vectorial capacity was introduced by Garrett-Jones (1964) and Garrett-Jones and Shidrawi (1969) as a means of singling out the vector components useful to entomologists in evaluating the potential ability of a particular insect population to spread a disease. In its simplest form, vectorial capacity is a function of female density relative to humans (*m*), biting frequency (*a*), survival rate (*p*), and duration of the extrinsic cycle (*n*), according to this simple expression:

$$C = \frac{ma^2p^n}{-\ln(p)}$$

Vectorial capacity is most sensitive to changes in survival rate (*p*) and biting frequency (*a*) of females. Because of their magnification by powers of *n* and *2*, respectively, even small changes result in large effects.

Vectorial capacity is oversimplified (Dye 1992), because in its original form it assumes perfect vector competence (see below). Yet in practice, even the simplest version of vectorial capacity is difficult to employ, because its parameters are not easy to measure. Still, this formula has tremendous heuristic value. It allows an entomologist to focus on control efforts that are likely to achieve maximum effect. For example, it becomes clear that the density of vectors, which can be manipulated by larval suppression, whether by insecticides or source reduction, is not nearly as important as reduced adult survival, whether by residual insecticide applied to resting sites, insecticide-treated bed nets, or reduced availability of sugar sources. It also shows, in a quantitative way, which coefficients are most important to measure and worth the effort to investigate by detailed study.

Thus, to increase its heuristic utility, researchers have suggested including an approximation of vector competence (*b*) as a factor in the numerator of vectorial capacity. In addition, to provide a more realistic value for the probability of survival, researchers have introduced survival rates as various sorts of non-linear functions (e.g. Bellan 2010, Dawes *et al.* 2009, Styer *et al.* 2007b), so that the probability of death may be high or low early in adult life, decline or gradually increase subsequently, then either increase greatly or decelerate at advanced vector age, based on field experiments (Harrington *et al.* 2008), meta-analyses of field data (Clements and Paterson 1981), and laboratory experiments (Dawes 2009, Styer *et al.* 2007a,b). All of these issues are discussed further, below.

Plant-affected components

The following features of vectorial capacity have been shown to be directly affected by plant feeding. The vector density (*m*) is indirectly affected through sugar feeding's influence both on reproduction (e.g. fecundity and male reproductive capacity) and on survival. The biting rate (*a*) is affected by sugar directly, through its influence on supplemental feeding within a gonotrophic cycle, its delaying of the primary (ovigenic) blood feeding while the crop contains a large sugar meal, and its delaying of oviposition. Survival (*p*) depends both on the frequency and quality of sugar feeding. Very little is known about whether energetic reserves affect host choice and the duration of the extrinsic cycle (*n*). Vector competence (*b*) is reported to be affected by the inclusion of sugar in mosquito diets, but the manner of the effect seems to depend on the vectors, pathogens, and plants involved. Flight activity and flight range also are vulnerable to plant-sugar availability, and their unfettered performance is an implicit assumption of vectorial capacity, so they also are mentioned below. A related assumption is that host use is random, though evidence suggests otherwise. Finally, a factor in age-dependent models of vectorial capacity is the age at which blood feeding commences (Styer *et al.* 2007a), which relates directly to the blood/sugar feeding choices of young females, discussed under 'Obligatory or facultative nature of sugar feeding'.

Vector competence

Here, we consider vector competence to be the product of the vector's characteristics, which will determine the success rate of a particular parasite to infect that vector, to develop, and then to

be transmitted to the extrinsic host (Hardy *et al.* 1983). There is a gauntlet of challenges parasites must run in order to infect and develop in the vector. Both the efficiency with which they do so and the strength of the challenge they face may be altered by environmental conditions, such as plant-sugar availability and hence energetic reserves of vectors.

The main challenges parasites face in their vectors are exposure to the proteinases and trypsins present in the midgut environment after ingestion with blood. For parasites spending a long period here (such as *Plasmodium* spp.), coagulation of the blood bolus and concomitant formation of the peritrophic matrix interfere with infection. The main bottleneck, however, is associated with invasion of the midgut epithelial cells and passage through the basal lamina to the abluminal side of the midgut. It is at this point that certain *Plasmodium* spp. are subject to melanization (e.g. Chun *et al.* 1995). Further challenges from the insect immune system are faced by parasites during migration through the haemocoel to either the lumen of the salivary glands or the head region (Beerntsen *et al.* 2000). Here we provide an overview of the ways in which plant feeding may affect these processes, in particular focusing on direct toxic effects of plant material on the parasite while it is in the midgut environment, on the effect of energy status on the immune response of the vector, and on the vector's energetic reserves, which serve as a nutrient source for the metabolic demands of the parasite as well as the defenses of the vector. It may be difficult to tease apart whether energy is expended on repair, immune function, or a different drain caused by the parasite.

Toxic effects on Leishmania *and* Plasmodium

The most immediate way in which feeding on particular plants could affect vector competence is by an inhibition of the infection by compounds present in the plant. To date, the most compelling evidence for the occurrence of a toxic effect on a parasite resulting from plant feeding is that of mortality and agglutination of *Leishmania major* Yakimoff and Schokhor, the cause of zoonotic cutaneous leishmaniasis, after plant feeding by *Phlebotomus papatasi* Scopoli (Jacobsen and Schlein 1999, Schlein 1986, Schein and Jacobsen 1994). This widespread sand fly inhabits semi-arid regions of the Mediterranean and Middle East and appears to depend on plants, using both their floral nectar and their tissue juices. Some of the host plants provide lectins and toxins. That these cause either agglutination or lysis of the parasites within the sand flies' midguts (Jacobson and Schlein 1999) became evident when sand flies artificially infected with promastigotes were kept for 7 days with access to branches of various plant species or to honeydew secretions. Seventeen percent of females with honeydew had decreased infections, whereas 35-65% with plants had reduced infection loads, compared to a sucrose control. After feeding on *Capparis spinosa* L., *Ricinus communis* L., or *Solanum luteum* Mill. many parasites were agglutinated in clumps and had disintegrated organelles or other aberrations (Schlein and Jacobsen 1994). Extracts of certain plants agglutinated *Leishmania* parasites *in vitro*. The inhibition of this toxic effect in *in vitro* assays was prevented by the presence of various sugars (Jacobsen and Schlein 1999). This result and that of prior studies indicate that lectins prevalent in plants agglutinate promastigotes of *Leishmania* spp. (Davidowicz *et al.* 1975, Dwyer 1974, Jacobsen *et al.* 1982).

Additionally, drought-induced sugar shortages in plants can affect vector population size and parasite survival. With short rasping stylets, the sand flies can cut into healthy plant tissues and ingest sugars, starch granules, and cellulose particles. They generate amylase in their saliva and elsewhere to digest the starch to simple sugars, and the parasites generate both amylase and glucosidase, the latter being capable of both cleaving sucrose and partially digesting cellulose (Jacobson and Schlein 2001, Jacobson *et al.* 2001). The resulting sugars benefit both sand fly and

parasite. During the summer, the plants are stressed by high temperatures and lack of rain, and the plants produce much less sugar. This causes shortened sand fly lifespans, thereby greatly reducing reproduction and also curtailing the probability that a female will become infected by feeding on an infected rodent, survive long enough to allow the parasite to complete its extrinsic cycle, and then transmit it to uninfected rodents. This effect appears to be offset by the natural selection for deprivation-resistant sand flies, which live longer under these conditions. The increased drought tolerance also has the side-effect of weakening the sand flies' ability to eliminate their parasite infections (Schlein and Jacobson 2001).

Vector-produced lectins, proteins that bind with a parasite's structural carbohydrates important for invasion, are implicated in the outcome of several other pathogen-vector associations. For example, in *Ae. aegypti*, addition of N-acetyl-D-glucosamine to a blood meal containing the filarial nematode *Brugia pahangi* Buckley and Edeson facilitated migration of the microfilariae into the haemocoel, apparently because this sugar blocked the action of gut lectins (Ham *et al.* 1991). Unknown at this point is whether sugars with similar effects may be present in plant nectar. Sugar meals have been reported to either enhance or retard the development of malaria and filariasis parasites within mosquitoes (Basseri *et al.* 2008, Kelly and Edman 1997, Pumpuni *et al.* 1996, Samish and Akov 1972, Vaughan *et al.* 1994, Weathersby and Noblet 1973), but these effects and their mechanisms have not been investigated in depth. It also has been reported that exposure of *An. gambiae* to some attractive plant species, before or after an infectious meal, greatly curtails the production of *P. falciparum* oocysts (Manda *et al.* 2005, 2007c, and pers. comm. to W.A.F.). This suggests that, as in sand flies, plant feeding by mosquitoes can affect vector competence directly.

Effects of parasites on sugar feeding (metabolic demands)

Parasitic infection with filarial nematodes or *Plasmodium* spp. reduces mosquito fecundity (Hurd *et al.* 1995), suggesting that mosquitoes harbouring such infections bear a considerable cost. For instance, a reduced egg output as high as 33% has been reported for *Aedes trivitattus* Coquillett infected with *Dirofilaria immitis* Leidy (Christensen 1981). The exact mechanism may be difficult to pinpoint, because the costs can be mediated either by the metabolic demands of the parasite or by the immune response of the vector to the parasite (Tripet *et al.* 2008). In either case, plant-sugar feeding may play an important role in compensating for energetic losses due to infection. An example of a parasite-mediated cost is an increased susceptibility to infection with bacteria following *Plasmodium* ookinete penetration of the midgut epithelium and development of the oocyst between the basement membrane and basal lamina of the midgut. One such bacterium is *Serratia marcescens* Bizio, which mosquitoes may obtain from contaminated sugar wicks in insectaries, which, in conjunction with *Plasmodium* infection, increases mortality strongly (Maier *et al.* 1987). This necessitates efficient midgut repair of invaded epithelial cells. In *An. stephensi* repair begins a few hours after infection with *P. falciparum* through the activation of nitric oxide synthase, resulting in apoptosis or necrosis of the invaded cells, and their subsequent extrusion and replacement. Besides the direct energetic cost, this process uses arginine, a dietary requirement of egg production, thus suggesting a nutrient conflict between fecundity and immune response (Tripet *et al.* 2008).

Evidence that growing oocysts rely on the energetic reserves of the mosquito is scant and mostly indirect. *An. stephensi* Liston infected with *Plasmodium cynomolgi* Mayer have reduced flight capability, as measured by their distance flown, speed, and duration of flight. Furthermore, pre-flight weight between uninfected and infected females was different, indicating that this could be due to differential use of carbohydrate reserves. This is supported by the intriguing finding that

isolated midguts of *An. stephensi* infected with *P. cynomolgi* used up to 8 times more glucose than controls over a 2-hr period (Schiefer *et al.* 1977). Similarly, flight muscles of *An. atroparvus* infected with *Brugia patei* Buckley, Nelson and Heisch were relatively depleted of glucose, and significant incorporation of amino acids by the filarial nematode was observed (Simpson and Laurence 1979).

Several investigators have examined how meals taken by adult mosquitoes prior or subsequent to infectious blood meals affect establishment of infection and development of parasites. Kelly and Edman (1996) provided *Ae. aegypti* with either a sucrose solution or water before an infectious (*Plasmodium gallinaceum* Brumpt) blood meal, and sugar or additional blood meals afterward. Oocyst counts were highest in the group with water before, and sugar after, the infective blood meal, but there were no significant differences in sporozoite load. Infectivity rate was lowest for females that had no access to sugar but had access to additional blood meals after infection, suggesting either that a lack of nutrients from sugar negatively affects parasite establishment or that subsequent blood meals and increased enzymatic activity in the midgut interferes with oocyst development. Vaughan *et al.* (1994), indeed, showed that the accelerated blood-meal digestion resulting from prior blood feeding in *An. gambiae* had a detrimental effect on the production of *P. falciparum* oocysts. Mosquitoes with access only to sugar developed the most oocysts, those with two prior blood meals the least. And this was evident only when ookinete abundance was low. However, when *An. gambiae* was fed blood from human volunteers naturally infected with *P. falciparum*, a different result was found (Okech *et al.* 2004). Mosquitoes that had two prior blood meals (4 days apart) had a higher infection rate than those with one blood meal or only a 10% glucose solution, but oocyst loads did not differ between treatments. Whether females were kept with sugar or just water for the first 2 days after emergence did not affect infection rates. The concentration of sucrose solutions provided to *Culex pipiens pipiens* L. did not affect susceptibility to West Nile virus, but a higher proportion of females transmitted the virus when low-concentration sucrose was available (Vaidyanathan *et al.* 2008).

Effects of energy state on the immune (melanization) response

A robust body of work exists on the insect melanization of parasites, a specialized component of the immune response that sometimes occurs during filarial nematode and *Plasmodium* infections. In a *Plasmodium*-refractory strain of *An. gambiae* (Collins *et al.* 1986) late ookinetes/early oocysts are readily encapsulated and melanized, and negatively charged C-25 Sephadex beads, when injected, elicit a very similar response. Due to the straightforward nature of this method for quantifying the level of response (the proportion of Sephadex beads melanized and the degree of melanization), and its proposed value as a general model for the strength of the immune response (Schwarz and Koella 2002), it can offer precise insights into the interactions between mosquito diet, energetic reserves, and immunity. Additionally, as with midgut repair in response to infection, the involvement of limiting resources besides energy reserves hints at an immunity-reproduction trade-off. For instance, l-tyrosine, an amino-acid precursor of melanin involved with egg-chorion tanning, might be diverted to melanization of a parasite. A case in point is *Armigeres subalbatus* Coquillett, which when challenged with *Brugia malayi* Brug experiences a delay of oviposition (Ferdig *et al.* 1993). Both the aforementioned arginine and tyrosine occur in nectar (e.g. in *Lantana camara* L. (Vrzal *et al.* 2010)), and thus it is plausible that nectar feeding positively mediates such trade-offs.

A number of studies have looked into factors such as age, prior adult diet, and larval nutrition on the efficacy of the adult melanization response, and they are briefly summarized here. In *Plasmodium*-susceptible or refractory laboratory strains of *An. gambiae*, melanization of C-25

beads was highest the day after emergence, and then dropped rapidly. In the two days following a blood meal, melanization was elevated in comparison to non-blood-fed mosquitoes of the same age in the refractory strain, whereas blood feeding had a negligible role in the response of susceptible mosquitoes (Chun *et al.* 1995). In the refractory strain, melanization decreased with increasing temperature and with restricted larval diet (Suwanchaichinda and Paskewitz 1998). In *Ae. aegypti,* melanization was positively correlated with age at pupation and with body size (Koella and Boëte 2002). This contrasts with the finding that *Ae. aegypti* refractory to *P. gallinaceum* had shorter development times and smaller body sizes than susceptible mosquitoes (Yan *et al.* 1997). Possibly, a different aspect of the immune response was involved in the latter study, which would indicate that nutritional status does not uniformly affect the immunocompetence of vectors. Body size did not affect melanization in *An. stephensi*, but adult diet did (Koella and Sørensen 2002). If females had taken a blood meal one day prior to being injected with a bead, the likelihood of complete melanization went up with increased concentration of the available sugar solution. However, if females had not obtained blood prior to injection, the proportion of females that completely melanized their beads did not depend on sucrose concentration. This suggests that to mount an effective immune response, females must feed from a high-quality sugar source to augment the energy and nutrients obtained from a blood meal.

An age-dependent aspect of the melanization-promoting effects of sugar and blood meals also has been found, both in laboratory and field-collected *An. gambiae* (Schwartz and Koella 2002). The strength of the melanization response decreased over the first 4 days of life. For very young females, an increase in glucose concentration increased melanization, but by 4 days this was no longer the case, and instead only a blood meal increased the immune response. Both the proportion of females melanizing beads and the intensity of their response was higher in field-collected specimens. Those with longer wings had a higher likelihood of carrying oocysts, and weak glucose solutions did not affect whether oocysts developed. Few females had melanized oocysts, suggesting that melanization is irrelevant for certain natural vector-malaria interactions. The authors concluded that sugar feeding by *An. gambiae* does not affect the immune response significantly, because young females will rely mostly on teneral reserves and older females on blood meals. However, this may be a premature conclusion if studies in laboratory cages misrepresent energetic expenditures in the field or if females that include sugar in their diet experience energetic increases over multiple gonotrophic cycles.

Clearly, the immune response is energetically costly, and there is limited evidence that this, rather than the metabolic demands of developing parasites, is the main metabolic burden borne by infected vectors. For instance, in the black fly *Simulium ornatum* Meigen infection with *Onchocerca lienalis* Stiles reduced ovarian vitellin contents by half, 36 hr after infection. Because this occurred even at very low levels of infection, it would seem that a costly immune response, rather than energetic drain by the parasite, is competing with fecundity (Hurd *et al.* 1995). Additionally, Rivero and Ferguson (2003) tested whether *Plasmodium*-infected *An. stephensi* females depleted their energetic reserves more rapidly than uninfected females. Levels of whole-body glycogen and lipid did not differ, but glucose amounts were much higher in females with developing oocysts. However, the number of oocysts present did not influence the amount of glucose, suggesting that *An. stephensi* increases its sugar intake when infected, irrespective of the infection load. A cost of infection unrelated to oocyst burden also was found for *An. stephensi* infected with two genotypes of *Plasmodium chabaudi* (Ferguson and Read 2002). Under conditions of sugar deprivation one clone *of P. chabaudi* was more virulent in the mosquito, despite producing a significantly lower oocyst burden than the other clone or a mixed infection. With *ad libitum* glucose, a mixed infection had the highest survivorship cost, and there was no difference between the single infections.

Virulence of different parasite genotypes therefore was concluded to depend on environmental circumstances (i.e. presence of sugar). Lambrechts *et al.* (2006) investigated how *An. stephensi* genotype and environmental conditions (differences in weak glucose solutions exposed on wicks) affected infection with *yoelii yoelii*. Infection rates did not differ among mosquito lines or glucose treatments, but, surprisingly, the number of oocysts was greater with access to 4% glucose than with 2% or 6%. Additionally, infected mosquitoes suffered higher mortality at low glucose concentrations than uninfected females did.

As indicated by the sometimes contradictory findings above, the manner in which sugar intake influences immunocompetence, infection rates, infection loads, and virulence is complex. The main point is that the parasite-vector relationship, and therefore possibly disease transmission in a given area, is highly specific to mosquito genotypes, parasite genotypes, sugar availability, and sometimes to sugar-by-genotype interactions. It is therefore important to place mosquito immune responses in an environmental context, and one aspect of environment (differing both geographically and seasonally) is the presence and abundance of attractive nectariferous plants. Thorough studies on natural host-parasite systems under semi-field conditions will be required before plant-vector-parasite relations can be elucidated and generalized.

Survival

Minor decreases in daily survivorship of adult mosquitoes result in stark declines in vectorial capacity. It is therefore vital to know how survival depends on an environmental factor that has not been taken into account in epidemiological models, namely the composition of the plant community. Few field studies have considered how different nectar sources and their presence affect mosquito longevity; most have been performed in laboratory or semi-field settings, whose results require careful extrapolation to the field. A focus of laboratory investigations has been the difference in survivorship of mosquitoes on diets of sugar and blood or on blood only.

Clements and Paterson (1981) reanalyzed survival data of mosquitoes and concluded that the common assumption of constant mortality throughout life rarely holds. Therefore, Mcdonald's (1957) malaria model assumption, that mosquito senescence in nature either does not occur or is entirely overshadowed by high daily mortality, also may not hold. Instead, a more realistic value for survival may be derived from a non-linear function, so that the probability of death increases at advanced age.

This is particularly relevant to vectorial capacity, because the age at which a female takes an infected blood meal then affects the probability of her living through the extrinsic incubation period. Models have been proposed that take this into account (Dawes *et al.* 2009, Rasgon *et al.* 2003, Styer *et al.* 2007a). Furthermore, different age-dependent mortality functions produce very different vectorial capacity outcomes. For example, a Gompertz model, where mortality increases exponentially after a certain stage, contrasts sharply with a logistic model, where mortality of older individuals decreases, resulting in a small but highly infective proportion of old mosquitoes (Carey 2001, Dawes *et al.* 2009). While it is established that sugar feeding increases longevity, equally important may be whether mortality of females with sugar in their diet is best described by a function that differs from that of females feeding on blood only. For *Ae. aegypti,* Styer *et al.* (2007a) found that this was not the case. Mortality functions best fit a logistic decline, whether the females had sugar only, blood only, or both. Okech *et al.* (2004) found that female *An. gambiae* kept on blood only senesced faster than females with access to sugar as well.

To date, few studies have delved into the survivorship of mosquitoes when allowed to feed on different natural plants, and these have mostly focused on the malaria vector *An. gambiae*. This is an oversight, given the wealth of knowledge concerning many non-vector taxa on how strongly plants differ in nectar rewards and secondary compounds, and how pollinators respond to plant cues and make use of these resources (Goulson 1999). As a result, basic behavioural investigations on the nectar-foraging behaviour of medically and veterinarily important taxa are just beginning. We do not know whether foraging vectors ignore certain plants, whether responses to plant volatiles are fixed or affected by individual experience, and whether certain vegetation types provide inadequate amounts of nectar.

First steps in this direction were cage studies of *An. gambiae* sugar intake and longevity, comparing cuttings of plant species that occurred in their natural habitat. Gary and Foster (2004) found that overnight females were mostly able to obtain sugar in comparable amounts from honeydew, castor bean (*R. communis*), and cassava (*Manihot esculenta* Crantz), whereas on lantana and on castor bean with the extra-floral nectaries covered there were no sugar-positive mosquitoes. Despite the similar amounts of sugar taken from honeydew, castor bean and cassava, mean survival times differed significantly, suggesting that other aspects of nectar composition affected mosquitoes. In a similar study, only castor bean increased survival to an extent comparable to a 6% sucrose control. Lantana and sweet potato (*Ipomoea batatus* Lam.) resulted in high percentages of sugar positive mosquitoes, but extended survival by an average of only one day over a water-only diet, likely due to the low amounts of sugar obtained. Access to one species (*Amaranthus hybridus* L.) resulted in a slightly worse survival than a water-only treatment (Impoinvil *et al.* 2004).

The sugar- and amino-acid composition of both floral or extra-floral nectar varies among plants and may influence survival of mosquitoes. Yet, how they do so has been studied only in laboratory settings. For example, Vrzal *et al.* (2010) assessed the effect of amino acids on survival of *Culex quinquefasciatus* Say males and females by adding certain amino acids to water or to a mixed sugar solution based on lantana nectar. Although adding amino acids to water did not increase survival, adding these amino acids to the sugar solution increased survival by 5% for females, but not males. Andersson (1992) tested longevity of female *Aedes communis* de Geer of different sizes and with access to different concentrations of sucrose or fructose. No difference in longevity was found between sucrose and fructose solutions, but the mean time of survival was shorter on 10% solutions than on the higher concentrations. Likewise, Fernandes and Briegel (2005) found that *An. gambiae* and *An. atroparvus* survived longest on 5-10% solutions, whereas Briegel *et al.* (2001) reported a linear relationship between sugar concentration and survival in *Ae. aegypti*, with 50% sucrose giving longest survival.

These results strongly suggest that mosquitoes should be discriminating in their plant feeding. That this is indeed the case was demonstrated by observing how frequently mosquitoes rested, probed, and fed on a group of thirteen different plant species in one large cage, and what percentage of mosquitoes had detectable sugar in the crop the morning after exposure to these plants. Based on these parameters, plants could fairly consistently be placed in preferred or non-preferred categories (Manda *et al.* 2007a). When the survival and fecundity of females feeding on these plants was evaluated (Manda *et al.* 2007b), of the plants that were consistently favoured by females, only *Parthenium hysterophorus* Buckl. did not significantly extend survival beyond that of a water-only control. After one blood meal, fecundity of females having fed on *P. hysterophorus* and lantana was less than that of those on the other plants, but after three consecutive blood meals there were no differences in fecundity.

Survival of female mosquitoes and sand flies allowed only blood meals, typically offered once a day, compared to those offered both blood and *ad libitum* sugar solution, has been thoroughly explored for a limited number of species, usually in laboratory cages. Sugar promoted survival of females in nearly every study (recent references: Bowen and Romo 1995b, Braks *et al.* 2006, Canyon *et al.* 1999, Costero *et al.* 1998, Day *et al.* 1994, Fernandes and Briegel 2005, Gary and Foster 2001, Kelly and Edman 1997, Manda *et al.* 2008, Okech *et al.* 2003, Schlein and Jacobson 1999, Straif and Beier 1996, Styer *et al.* 2007a, Xue *et al.* 2008, 2010). In some studies the effect of sugar deprivation on survival may have been exacerbated by restricted access to blood (Okech *et al.* 2003, Souza-Neto *et al.* 2007). Most studies indicate that survival on a mixed sugar-and-blood diet is greater than on sugar alone (Styer *et al.* 2007a, Xue *et al.* 2008, 2010), though Joy *et al.* (2010) found that lifespan was increased by restricted access to blood, so that female *Ae. aegypti* offered one blood meal or none lived longer than females offered blood once a week.

Biting frequency

As noted above, nearly all studies indicate that sugar availability reduces mosquito blood-feeding frequency. The most recent investigations confirm that sugar deprivation tends to promote blood feeding or makes females more responsive to host stimuli or less deterred by repellents (Bowen and Romo 1995a,b, Bowen *et al.* 1995, Braks *et al.* 2006, Canyon *et al.* 1999, Fernandez and Klowden 1995, Gary and Foster 2001, Renshaw *et al.* 1995, Takken *et al.* 1998, Xue and Barnard 1999, 2008). This effect probably is the manifestation of one or more of these phenomena: (a) crop sugar raises the threshold of responsiveness to blood-host stimuli, (b) a large energy reserve depresses the need for supplemental blood feeding during blood digestion, and (c) either crop sugar or a large energy reserve reduces activity and consequently delays oviposition, thus retarding the initiation of each new gonotrophic cycle. The overall result, taken by itself, suggests that the absence of sugar sources increases vectorial capacity. However, all of these studies were conducted in the laboratory, where abnormal amounts and frequencies of sugar ingestion may prolong the interval between blood meals. Field evidence for sugar's depressing effect is equivocal. *Aedes provocans* Walker females with large amounts of sugar generally were at rest, those with moderate meals were biting, and those with small meals were nectar feeding (Smith and Gadawski 1994). Resting *Anopheles freeborni* Aitken more often contained plant sugar than those seeking blood hosts (Holliday-Hanson *et al.* 1997), similar to earlier field work on salt marsh mosquitoes by Magnarelli (1978, 1979, 1980). Female *Ae. aegypti* released after receiving both *ad lib* sugar and replete blood meals completed their gonotrophic cycles 2 days later than those receiving only blood (Morrison *et al.* 1999). An exceptional result was reported by Gu *et al.* (2011), who found that *An. sergentii* in an oasis with a prominent sugar source had shorter gonotrophic cycles over much longer life spans than a population at a similar oasis without that sugar source. A similar conclusion was drawn from field data by Gadawski and Smith (1992). However, in the latter case preliminary sugar meals probably were necessary simply to hasten sexual maturation and initiate the blood-feeding mode.

Energetic reserves resulting from larval feeding may affect biting rates in different ways than those resulting from sugar feeding. For instance, mosquitoes emerging from nutritionally poor larval sites typically are smaller and show reduced host responsiveness. Thus, low energetic levels initially may favour sugar feeding and depress biting rates. On the other hand, small females often require an additional blood meal to bring their ovaries to the pre-vitellogenic resting stage. It has been suggested for both *An. gambiae* and *Ae. aegypti* that energetic deficits are compensated by supplementary blood meals. Such additional, gonotrophically discordant, blood meals increase vectorial capacity. The relative importance of both reduced host-responsiveness and the need for supplemental blood meals thus has implications for the effect of body size on vectorial capacity.

To elucidate, we will review the effects of sugar feeding and energetic reserves on components of biting rate: host responsiveness, pre-vitellogenic and supplementary meals, oviposition delay, and biting persistence.

Host responsiveness

Several studies have investigated the initiation of responsiveness to blood hosts in poorly nourished mosquitoes. Female *Ae. aegypti*, reared on a low diet, showed a weaker response to a host odour than did those on a standard diet (Klowden *et al.* 1988). Access to a 1% or a 10% sucrose solution did not mitigate this weakness. *Aedes bahamensis* Dyar and Knab developed sensitivity to blood hosts faster if they received more nutrition as larvae (Bowen and Romo 1995b). Initiation of host-seeking in *Aedes cantans* Meigen and *Aedes punctor* Kirby was related to accumulated lipid reserves, either through larval feeding or nectar feeding. Whereas *Ae. cantans* would not take a blood meal before 192 hr post-emergence, *Ae. punctor* synthesizes lipids more rapidly and was willing to blood feed after 48 hr (Renshaw *et al.* 1995).

Wing lengths of newly emerged *An. gambiae* were smaller than those of the host-seeking population in the field (Lyimo and Takken 1993), suggesting that a substantial portion of small females dies between emergence and host location, or never expresses host-seeking behaviour. Takken *et al.* (1998) found that responsiveness of large and small females to a human hand in an olfactometer increased similarly over the course of 6 days, but large-bodied females were always more responsive, despite access to 10% sucrose to both size classes. Similar body-size differences in olfactometer performance during attraction to a sugar source one day after emergence have been observed (Foster and Takken 2004). These results indicate that small-bodied females are debilitated by more than just a small energy reserve.

Supplementary blood meals and ovarian development

The taking of multiple blood meals per gonotrophic cycle has most notably been observed and linked to the vector status of *Ae. aegypti* and *An. gambiae* (Beier 1996, Norris *et al.* 2010, Scott *et al.* 1993). Multiple blood meals may be taken by very young gonoinactive females as 'pre-gravid' meals, typically in small females that developed in nutritionally poor larval habitats (Feinsod and Spielman 1980, Lyimo and Takken 1993) or may be taken later in life, either as supplementary meals (e.g. Foster and Eischen 1987) or as primary meals that initiate overlapping gonotrophic cycles (Briegel and Hörler 1993). The influence of teneral reserves and reserves accumulated through sugar feeding on either type of multiple feeding may be different. For instance, female *Ae. aegypti* kept with *ad libitum* sugar show host-seeking inhibition during oogenesis (Klowden and Lea 1979), but this is not the case when lacking sugar (Klowden 1986), as a large proportion of gravid females then continues to seek a host. This depressing effect of sugar is also reflected in biting rates. For example, *Ae. aegypti* kept with only water had a higher biting rate, explained mainly by an increased frequency of supplementary feeding, than did females kept with honey. The opposite effect occurred in *Anopheles quadrimaculatus* Say when kept on water. These females had a supplementary feeding rate almost equal to those on honey, but a higher total biting rate, likely due to the absence of a variable delay in oviposition caused by honey feeding (Foster and Eischen 1987). Supplementary feeding appears to be more common among some tropical anophelines, perhaps used as a tactic to compensate for a smaller blood meal capacity and low teneral reserves, with additional meals increasing fecundity and maternal deposits (Briegel and Hörler 1993). In *An. gambiae*, sugar availability after blood feeding does not inhibit the host-seeking response in the same way as it does for *Ae. aegypti*. While the response to a host was

inhibited for 12 hr following a blood meal, after 24 and 48 hr females showed no sign of inhibition (Klowden and Briegel 1994). Straif and Beier (1996) found that it was only the older females (>20 days) that showed an increased biting rate when kept in the absence of sugar. That was not due to an increase in biting rate with age, but rather to a subgroup of the sugar-deprived females, which fed more often and therefore survived longer.

Female body size does not appear to affect this sugar-dependent biting inhibition, or its absence, prior to oviposition, because gravid females reared under either standard or deficient larval conditions, then kept with sugar, do not respond to hosts (Klowden *et al.* 1988). Thus, either body size has no impact on the tendency toward supplementary blood meals later in life, or the energy deficit required to lower the host-seeking inhibition can be compensated by sugar feeding. This does not rule out the possibility that small females without sugar may take more supplementary blood meals. In the field, Scott *et al.* (2000) found a negative relationship between wing length and blood-feeding rate of *Ae. aegypti* in Thailand, but not in Puerto Rico. Geographic variation in gonotrophic discordance is also apparent among anophelines (Beier 1996, Norris *et al.* 2010). Multiple feeding appeared to be more common in a highland site in western Kenya in *An. gambiae s.s.* and *An. funestus* (14, 11%) than in a lowland site (0, 2%) (Scott *et al.* 2006). The cause of such differences is unknown, but may involve local population adaptation, nutrition, temperature, or all three.

Smaller, nutritionally deprived *An. gambiae* females often require one or two additional blood meals before their primary ovarian follicles reach the (gonoactive) resting stage. It is not entirely clear whether sugar feeding can make up for this nutrient deficit, and how size and sugar feeding influence biting tendencies at this time. Takken *et al.* (1998) showed that despite their lower responsiveness to hosts, small females are in great need of blood. Small females, despite access to sugar, did not reach the resting stage without a blood meal, whereas 52% of large females did. In another study, sugar was able to substitute for a non-vitellogenic blood meal. With sugar, females of all sizes were able to mature eggs with just one blood meal (Fernandes and Briegel 2005).

In summary, while small female mosquitoes may have an increased need for blood meals soon after emergence to make up for energetic deficits, their decreased sugar-and-blood-host-seeking capabilities interfere with this, resulting in a higher probability of feeding on sugar, if sugar is more accessible. Sugar feeding inhibits supplementary feeding in some species, but does not do so in *Anopheles*. Finally, small size may increase the tendency to take supplemental blood meals during later gonotrophic cycles, but this is not yet established.

Delay of oviposition

De Meillon *et al.* (1967) discovered that access to cane sugar resulted in erratic and delayed oviposition by *Culex pipiens*. Likewise, inseminated female *Aedes vexans* Meigen with access to sugar spread their oviposition over a slightly longer period, more often failed to oviposit, and retained a greater number of eggs (Shroyer and Sanders 1977). Increased amounts of egg retention by females given access to sugar after blood feeding also was reported for *Anopheles nuneztovari* Gabaldón (Lounibos and Conn 1991). Klowden and Dutro (1990) found that sugar feeding reduced the responsiveness of *Ae. aegypti* to oviposition-site stimuli and this inhibition was greater at higher concentrations of sugar. This effect was attributed to reduced flight activity of sugar-fed mosquitoes. However, it is plausible that the delayed oviposition when sugar is abundant may be an adaptive response to a favourable energy state, i.e. females with a greater flight range and higher prospects for survival may be more selective in their choice of oviposition

sites and distribution of eggs among them. Females that are close to starvation may readily accept oviposition sites of lesser quality. For example, Tsunoda *et al.* (2010), investigating the effects of body size and sugar access on skip oviposition by *Ae. aegypti*, found that sugar delayed the time between blood feeding and peak oviposition and stretched out the period of egg-laying. Additionally, both larger and sugar-fed females oviposited higher above the water surface and oviposited in a greater number of cups over the course of 8 days. An advantage of this behaviour is that hatching of larvae will be more varied in space and time, a bet-hedging tactic.

Persistence

A special aspect of blood feeding that also is influenced by plant sugar is a vector's persistence at a host. Persistence probably has some bearing on the interval between successful blood meals or infectious bites. Differences in persistence in obtaining a primary blood meal have been detected in mosquitoes that differ in their energetic state as a result of access to sugar. It is not always clear whether this is an effect of sugar in the crop, energy reserves in the fat body, haemolymph, and flight muscles, or all of them. Attack duration declined with repeated attacks by *Aedes triseriatus* Say and *Ae. aegypti,* but it declined more rapidly in females without sugar (Walker and Edman 1985, Nasci 1991). In an *An. gambiae* study that distinguished between persistence (time elapsed before resting on a wall for more than 3 min) and attack number (number of host landings per second), only the number of attacks depended on energy state (Roitberg *et al.* 2010). Females never deprived of sugar had the highest number of attacks. The number of attacks was lowest in those deprived one day and somewhat higher at 2 days, suggesting that feeding tactics vary in a non-linear way with changing energy status. Whether these effects influence biting frequency over multiple gonotrophic cycles remains to be examined.

Reproduction and population density

Effects of sugar feeding and energetic reserves on fecundity

The importance of sugar feeding to a vector's reproduction has been evaluated primarily by laboratory studies, though field experiments are gaining prominence. We follow the definition of fecundity as the number of gametes produced by an individual over its lifetime, and fertility as the number of viable offspring produced (Clements 1992). The latter would be affected by factors such as egg retention, viability, and fertilization, whereas fecundity, which we consider here, is simply the number of mature eggs developed.

Blood meals are used both for vitellogenesis and extra-ovarian reserves (Briegel 1990). The proportion used for oogenesis differs with female body size, suggesting flexibility in the allocation of nutrients. When females are close to starvation or emerge with low teneral reserves, they prioritize synthesizing extra-ovarian reserves. With higher energy levels, a greater investment in vitellogenesis is possible. Increasing lipid reserves by carbohydrate feeding should thus increase egg-batch size of females. Laboratory experiments have demonstrated that female mosquitoes with access to sugar usually produce more eggs per gonotrophic cycle (Briegel *et al.* 2002, Foster and Eischen 1987, Harrington *et al.* 2001, Manda *et al.* 2007a, Mostowy and Foster 2004, Gary and Foster unpublished data). Among first-cycle autogenous species there are exceptions to sugar's positive effect on egg-batch size (e.g. Telang and Wells 2004). The timing of sugar feeding also matters. *An. nuneztovari* that had continuous access to sugar or were deprived of it after blood feeding did not differ in their total fecundity (Lounibos and Conn 1991), indicating that feeding on sugar after a blood meal has little effect on egg production during that gonotrophic cycle.

But some mosquitoes may rely heavily on sugar feeding during vitellogenesis. For instance, *Ae. communis* females lacking access to sugar after a blood meal failed to develop follicles to Christopher's stage V (Andersson 1992).

Directly after taking a sugar meal, a female's distended crop will compete for abdominal space with the midgut's future blood meal, resulting in a smaller blood meal. Because blood-meal size correlates with fecundity, the contribution of sugar feeding to fecundity will depend on the digestion rate and the interval between the sugar and blood meals. This was shown for *Ae. aegypti*, where females with high levels of energy reserves, but with an empty crop, had the highest fecundity, and females with low reserves but a full crop the lowest fecundity. Females with high reserves and a full crop or low reserves but an empty crop had similar, intermediate fecundities. Only in low-reserve females with empty crops was the conversion of blood into eggs considerably less efficient. Thus, in sugar-deprived females fecundity appears to be limited by energy levels, whereas in sugar-fed females fecundity appears to be limited mostly by blood-meal size (Mostowy and Foster 2004).

Fitness

A case has been made, based on the infrequency of sugar feeding and high proportions of supplementary blood meals taken by the anthropophilic species *Ae. aegypti* and *An. gambiae,* that sugar is an inconsequential component of their diet where human hosts are common, and that females optimize their fitness by feeding exclusively on blood. This strict interpretation does not necessarily follow from field data showing low levels of fructose positivity, because sugar may be taken more frequently farther away from indoor sampling sites or during times of stringency. Even if sugar feeding is indeed uncommon in the field, it is not necessarily unimportant: the reproductive success of females that seldom feed on sugar may be higher than that of females that never do so (Ma and Roitberg 2008). Otherwise, it would imply these mosquitoes either are not behaving optimally (i.e. they make mistakes), or the selective pressures toward a blood-only diet are operating, but exclusive blood feeding has not yet reached fixation in certain populations.

Here, we briefly define the measures of fitness that encompass several life history parameters that are affected by sugar feeding. The most commonly used measure of fitness is Fisher's Malthusian parameter, r, the intrinsic rate of increase. For age-structured populations r is obtained by solving the characteristic equation (Roff 1992):

$$\int_0^\infty e^{-rx}\, l(x)\, m(x)\, dx = 1$$

where $l(x)$ is the proportion of the cohort alive at age x, and $m(x)$ the production of female offspring at age x. Charlesworth (1994) described this as an adequate measure of fitness in the case of weak selection and random mating with respect to age in density-independent and constant environments. If r is close to zero (the population is stationary), the use of R_0 may be justified. R_0, the net reproductive rate, is the expected number of female offspring produced by a female over her lifetime:

$$R_0 = \sum_0^\infty l(x)\, m(x)$$

It has been argued that in order for R_0 to be a useful indicator of fitness, the growth rate of the population should not just average to zero, but actually be zero (i.e. it would not be a suitable measure for fluctuating populations) and therefore is better seen as one component of fitness

(Caswell 2002). In environments that are not constant (e.g. alternating 'good' or 'bad' years, or seasons) the geometric mean of the finite rate of increase has been proposed as the most accurate indicator of fitness (Roff 1992).

Whether females of anthropophilic mosquitoes have a greater or lesser fitness with access to blood exclusively, or to blood and also *ad libitum* access to sugar, has been the subject of several studies. An overview of these, and of how including sugar in the diet affects different fitness parameters, is given in Table 1. Most studies report values for daily survival (l_x) and fecundity (m_x), and fitness measures r and R_0. The only published life-table data for *An. gambiae s.s.* to date reported that both r and R_0 were slightly higher in cages without sugar (Gary and Foster 2001). Braks *et al.* (2006), comparing *Ae. albopictus* to *Ae. aegypti*, found a similar response to the diets in both species. All other studies we are aware of have focused on *Ae. aegypti*. While there is a general consensus that survival is increased when sugar is available, and daily fecundity is decreased, not all studies bear this out. In one, survivorship decreased when females could feed on sugar (Scott *et al.* 1997), and others reported no significant differences between the diets (Canyon *et al.* 1999, Naksathit and Scott 1998). Styer *et al.* (2007b) found no difference in survival when females were housed individually, but an increase with sugar when kept in cages of 200. Harrington *et al.* (2001) found that the outcome depended on the blood source, i.e. with human blood, sugar access made no difference, but with mouse blood, survivorship increased with sugar availability.

The effect of sugar feeding on daily fecundity was mostly negative in these studies, with some exceptions (Day *et al.* 1994, Scott *et al.* 1997). When blood was offered daily, Styer *et al.* (2007b) found no difference in the daily egg production of females offered blood or both sugar and blood. When blood was offered every 2 days, however, females with access to sugar had a significantly lower daily number of offspring. This may have been due to increased reliance on sugar feeding – the infrequency of blood having resulted in missed opportunities to blood feed or the ingestion of smaller blood volumes.

The net reproductive rate, R_0, was lower for *Ae. aegypti* females with access to sugar in the majority of these studies. Braks *et al.* (2006) and Day *et al.* (1994) reported no difference in this parameter. The only study in which sugar increased R_0 occurred when females were housed in cages together, instead of individually (Styer *et al.* 2007b). Crowding may have increased levels of disturbance, causing more flight activity and energy consumption. Whether those conditions are a better reflection of the stress factors faced in a more natural setting is debatable. The intrinsic rate of increase most often decreased when sugar was available. Costero *et al.* (1998) reported different results for cool and hot seasons in Puerto Rico. During the hot season females feeding only on blood had the advantage, whereas there was no difference in r during the cool season. In crowded cages r was higher when females had daily access to sugar and blood, but, counterintuitively, lower when they had access to blood every 2 days (Styer *et al.* 2007b).

The general impression is that in the laboratory the prolonged life of a sugar- and human-fed female is often insufficient to offset sugar's negative effect on lifetime fecundity in these anthropophilic species. On the face of it, the depressing effect of sugar on long-term fecundity means that natural selection should favour the absence of sugar feeding in these females. This selection should be particularly strong in species lacking a quiescent egg stage (i.e. are unable to accumulate offspring in an 'egg bank' and engage in installment hatching after receiving hatching stimuli), during periods of population growth. Quiescent eggs occur in *Ae. aegypti* and other aedines, but only to a very limited extent in *An. gambiae* and other anophelines. Therefore, anophelines should take full advantage of opportunities for unrestricted reproduction during

Table 1. Summary of published effects of a blood and sugar diet, compared to blood-only diet, on fitness components of mosquitoes.

Study	Species	Setting	Sugar solution % (*ad libitum*)	Blood host	Blood host availability/d	Biting rate	Survival (L_x)	Fecundity (M_x)	R_0	r	Comments
Straif and Beier 1996	*An. gambiae*	Glass jar, individually	10% (all ♀'s for 1st 4 days)	Mouse	15 min	↓ (1)	↑	–	–	–	(1) only for oldest age group
Gary and Foster 2001	*An. gambiae s.s.*	Cage, individual & pooled	10% (all ♀'s for 1st 2 days)	Human	10 min	↓ (1)	↑	↓	↓ (2)	↓	Vectorial capacity ↓ (1) difference bigger in older ♀'s (2) slightly?
Scott *et al.* 1997	*Ae. aegypti*	Cage, individual, ambient	10%	Human	10 min	–	↓	↔	↓	↓	
Braks *et al.* 2006	*Ae. albopictus* and *Ae. aegypti*	Cage, individually	10%	Human	10 min	↓	↑	↓	↔	–	Same response for both species
Naksathit and Scott 1998	*Ae. aegypti*	Cage, individually	10%	Human	10 min	–	↔	↓	↓	↓	Same pattern for large & small ♀'s
Harrington *et al.* 2001	*Ae. aegypti*	Cage, individually	20%	(1) Human (2) Mouse	15 min	(3)	(1) ↔ (2) ↑	↓? (3)	↓? (3)	↓?	(3) no statistics provided
Styer *et al.* 2007	*Ae. aegypti*	Cage, 200 ♀ + ♂'s	10%	Human	(1) 15 min every other day (2) 10 min	–	↑	(1) ↓ (2) ↔	(1) ↑ (2) ↑	(1) ↓ (2) ↑	
		individually			10 min (?)	–	↔	↓	↓	NM?	
Costero *et al.* 1998	*Ae. aegypti*	Cage, individually, ambient	10%	Human	10 min	– (?)	↑	↓	↓	(1) ↔ (2) ↓	(1) cool season (2) hot season
Day *et al.* 1994	*Ae. aegypti*	Cage, 200 ♀ + 200 ♂'s	2%	Chicken	2 hrs	–	↑ (1)	↔	↔	–	(1) mean LT_{50} & LT_{90}
Canyon *et al.* 1999	*Ae. aegypti*	Specimen vial, 5 ♀, 1 ♂	3% & 10%	Human	10 min, 4×	↓	↔	↓	–	–	High levels of egg retention, exp. stopped at 12 d

Arrows refer to the effect on parameter by including sugar in the diet compared to blood only: ↓ = decrease; ↑ = increase; ↔ = no difference; – = not measured.

periods of population growth, by feeding only on blood. Yet, they do feed on sugar, both in the lab and in the field.

Perhaps the main critical question about these studies is whether the conditions were representative of those faced by mosquitoes in nature. A mark-release-recapture experiment in Puerto Rico, where female *Ae. aegypti* were kept either with blood only or with blood + sugar for 5 days, then released, did support the general hypothesis that anthropophilic females should rarely feed on sugar (Morrison *et al.* 1999). However, additional validation would be useful, as in this study values for *r* and R_0 were not obtained, fecundity was assessed for only one gonotrophic cycle, and survival was assessed for just 5 days. Possibly the difference in environment between nature and laboratory cages does not matter, but the higher energetic expenditures and greater risk of mortality associated with real situations – evident even in mesocosm-vs.-cage comparisons (Stone *et al.* 2009, 2011) – could enhance the reproductive success of females that do take sugar. In small spaces, flight energy may be consumed at a lower rate, reducing demand for sugar, yet sugar is encountered incidentally but frequently in cages and ingested after stimulatory contact. Excessive sugar feeding may exacerbate its negative effects on the volume of blood meals, the frequency of blood feeding, and prompt oviposition. More intangible aspects need further study, too, including location of higher quality oviposition sites (Tsunoda *et al.* 2010), mate choice (Stone *et al.* 2011), and maternal effects (Fernandes and Briegel, 2005).

In zoophilic species, whether aedine, culicine, or anopheline, the reduced-survival penalty of relying on blood as the sole source of energy appears to be much greater. Though not well documented, the ability of blood (either human or animal) to sustain life in the absence of sugar appears to be much poorer in these animal-feeding mosquitoes (Fernandes and Briegel 2005, Nayar and Sauerman 1971, Wittie 2003), probably because they are less able to cope with the costs of protein catabolism.

Male insemination capacity and competitiveness

Males often are completely overlooked in studies of nutrition's effects on survival and reproduction. But interest in the biology of male mosquitoes has increased, largely related to concerns about the competitiveness of sterile or genetically manipulated males compared to wild-type males. Experience has shown that competitiveness can be a pitfall for genetic control programs (Reisen 2004).

Due to the commonly held assumption that in polygynous species – in which the operational sex ratio of male to female will be high (Emlen and Oring 1977) – all females will become inseminated, repercussions of male mating behaviour on population dynamics have not been well studied. Assuming that males can inseminate multiple females, regardless of food intake, probably adds to this neglect. A recent review of entomological field studies reports that mating failure of females in nature is common (Rhainds 2010). Often this is age-related (i.e. 'temporary wallflowers' rather than total mating failure), so that less-preferred females take longer to become inseminated, lowering their fitness. A male preference for large female mosquitoes (Okanda *et al.* 2002) and those exhibiting certain qualities or bearing species-specific characters (Hancock *et al.* 1990, South and Arnqvist 2011) has been reported.

Mating failure of mosquitoes in nature is difficult to assess because of the lack of a convenient age-grading method that does not rely on female reproduction. However, because males do not ingest blood, their sexual responsiveness, flight activity, and survival – and consequently their

insemination potential – is completely dependent on reserves carried over from larval feeding and from post-emergence sugar feeding. Hence, changes in sugar availability may lead to pronounced shifts in the operational sex ratio. If male population size declines sufficiently, an Allee effect (i.e. at low numbers, there is a positive relationship between population growth rate and population density) will occur in certain environments or seasons. This requires that the insemination rate of females will drop with declining male:female sex ratio. This will depend largely on the upper bounds of male mating capacity and on the efficiency with which females locate large swarms. Howell and Knols (2009) recently reviewed the mating biology of male mosquitoes and suggested that a typical anopheline male may mate 0-3 times in its lifetime (in monandrous species with a 1:1 sex ratio, which is typical of mosquitoes, the average must be 1), but the maximum probably is higher.

To have even a chance at reproductive success, males must survive through a period of maturation when their terminalia rotate and antennal fibrillae can become erect. Following this they have to engage in the energetically costly and risky behaviour of swarming. In most species this takes place at dusk or both dusk and dawn, though many aedines swarm during the day. Swarming typically lasts for only 10-30 min, but sometimes for hours, especially at higher latitudes. The swarm itself is stationary, with males engaging in a constant to-and-fro, up-and-down movement (Downes 1969), until a female is encountered and clasped. Having sufficient energy to perform this behaviour for multiple nights would clearly favour a male's prospects of mating. Furthermore, a male's mating ability and insemination capacity improves during his first week of adult life (e.g. Verhoek and Takken 1994).

Studies of the effect of body size, an indicator of the reserves a male accumulated as a larva, on mating performance are inconsistent. This may be because on the one hand a larger male is likely to have increased longevity, increased ability to locate a swarm site, and perhaps an increased duration within the swarm (Yuval and Bouskila 1993, Yuval *et al.* 1994). On the other hand, a larger male may have poorer agility in flight and decreased competitive ability to grasp a female (Ng'habi *et al.* 2008). Thus, the ability of male mosquitoes to locate and feed on sugar throughout their lives may be the prime determinant of mating success. Yuval *et al.* (1994) concluded that *An. freeborni* feed on sugar only after swarming in the evening, because only resting males collected in the morning contained significant proportions of fructose, whereas males collected in the late afternoon or during swarming did not. The same appears to hold true for *An. gambiae* (Stone, personal observation) and *Culex tarsalis* Coquillett (Reisen *et al.* 1986). The amount of sugar and glycogen, but not lipids, decreased significantly from the start to the end of swarming. The energetic cost of swarming was calculated to be 0.39-0.51 cal/h, resulting in a consumption of over 50% of available reserves if a male were to swarm for 40 min (Yuval *et al.* 1994). In the case of *Ae. aegypti*, enough males may survive in the absence of sugar to inseminate all females in the same age cohort (Braks *et al.* 2006), at least among mosquitoes that have developed under ideal conditions and are held in small cages. Yet natural selection should always favour males that take sugar meals and thereby greatly increase their mating potential and competitive abilities.

For *An. gambiae* males, successfully mating with a female in the absence of sugar is an almost insurmountable task. Increased teneral reserves and low environmental temperature, causing lengthened survival, increase the odds somewhat. Among sugar-fed males, Gary *et al.* (2009) found that the percentage that erected fibrillae and swarmed increased to almost 100% over the course of 2-3 days, whereas without sugar the percentage doing so was already much diminished by the second day. The proportion of females that were inseminated was influenced by male body size, cage size, and temperature; in small cages sugar deprivation did not affect insemination rates

for the first 2 days of cohabitation, but a smaller proportion was inseminated after 3 days at 23 °C when sugar was absent (at 27 °C all males had died by this time). In a follow-up study, the effect of sugar availability on insemination rates of females in more natural, energetically demanding mesocosms was studied with overlapping cohorts of males and females (i.e. groups of 0-day old males and females were released for 10 consecutive days). Maturing females would therefore have multiple opportunities to mate with maturing males. After 10 days, the cumulative insemination rate of females when sugar was present was 49.7%, compared to 10.9% in the absence of sugar (Stone *et al.* 2009). As mentioned above, directly related to insemination rates is the amount of time a female is likely to remain unmated. If this window between female maturity and time of actual mating is expanded, females will suffer a fitness cost, and this will be absolute if they fail to become inseminated. If this occurs for a significant proportion of females, this may have population-level repercussions. Indeed, simulations of a population projection matrix show just that. When sugar sources are removed from the environment, population sizes are reduced to zero over a wide range of life history parameters (e.g. fecundity), suggesting that *An. gambiae* populations are not viable in the absence of sugar sources (Stone *et al.* 2009). It would be valuable to gain deeper insight into the link between male survival and mating ability and their foraging behaviour. Particularly relevant is how well males are sustained on poor-to-medium-quality sugar hosts, and to what extent males can make up for this by increasing their foraging efforts.

Flight activity and range

Although not a coefficient of vectorial capacity, flight range is important to transmission. This is partly because vectorial capacity makes the assumption that biting will be random within the vertebrate host population. As flight is restricted, chances increase that vectors will bite the same hosts repeatedly, introducing the complications of redundant infections or superinfections. Flight range also is critical to the successful movements of vectors between oviposition sites and vertebrate hosts when the two are widely separated (e.g. Clarke *et al.* 2002). These transmigrations must be supported by energy derived either from portions of blood meals not used in vitellogenesis or from plant-sugar meals, as flight-mill studies have shown (e.g. Kaufmann and Briegel 2004). They almost certainly affect two critical vectorial capacity components: blood-feeding frequency and survival.

Learning

The availability of sugar, and therefore its effect on biting frequency and survival, probably changes with vector age as a result of experience. If this conjecture is valid, then sugar's effects on vectorial capacity also change with age. Early experiments with *Cx. quinquefasciatus* and *Cx. pipiens* demonstrated that, by associating plant-produced volatiles with the presence of sugar, young adults became more responsive to them (Jhumur *et al.* 2006, Tomberlin *et al.* 2006). This learning ability is expected to be advantageous in environments where sugar production by different plant species changes seasonally and also would allow adjustment to different plant communities. Non-random selection of blood hosts (McCall *et al.* 2001) and oviposition sites (McCall and Eaton 2001), as a result of experience, also may cause distortions unaccounted for in assessments of vectorial capacity (McCall and Kelly 2002).

Plant-based techniques for vector control and interruption of pathogen transmission

Reducing the incidence of malaria, the deadliest among mosquito-borne diseases, relies on reducing the entomological inoculation rate (EIR), the number of infectious bites received per person per unit of time, usually one year. This relies on the biting rate, *a*, and density, *m*, of the vector population, as well as the sporozoite rate, i.e. the proportion with sporozoites in their salivary glands. The main vector control methods used to prevent malaria are currently indoor residual spraying (IRS) and insecticide-treated bed nets (ITN). While great reductions in EIR are often achieved with these methods, the only recent control efforts that have produced an EIR<1, the level required to achieve a sustained reduction in infected-case prevalence, combined ITNs with source reduction (Shaukat *et al.* 2010), i.e. one form of integrated vector management (IVM). The effectiveness of IVM, the WHO-recommended approach to vector-borne disease control (WHO 2004), comes from combining two or more methods that are most efficacious in a particular setting and that complement each other synergistically. Novel control methods that can be used in IVM are urgently needed, and those targeting components of the mosquito life cycle that are left untouched by current methods may be especially promising (Ferguson *et al.* 2010). Because sugar feeding may be exploited for such a purpose, it is all the more poignant that basic knowledge of the feeding decisions and behaviour of even the most important malaria vectors remains scant to date. Here we review promising studies that make use of the sugar feeding behaviour of mosquitoes in control and surveillance of pathogens and vectors.

Marking

Mosquitoes and sand flies can be marked to study their behaviour and survival. One efficient method is to allow the mosquitoes to feed on sugar that has been mixed with a dye or radioisotope. An advantage is that the vectors mark themselves, either at emergence (Reeves *et al.* 1946, Midega *et al.* 2007) or at a suspected host plant (Abdel-Malek 1964, Abdel-Malek and Baldwin 1961, Müller and Schlein 2006, Müller *et al.* 2010b, Schlein and Müller 2008, Schlein and Pener 1990), thus avoiding the disruptive effects of handling. Marking has been effective in experiments on dispersal, flight range, survival, and plant-host utilization. An unfortunate side-effect of field studies in which a marked sugar solution is provided right at the place of adult emergence is that the vectors have an unnatural, easily accessed, and very early source of energy. Therefore, such studies may produce misleading data on the timing of mating, gonotrophic cycle events, early mortality, and average distance flown from the emergence site.

Trapping and surveillance of vectors, and detection of pathogens

Plant-based attractants

Vectors find their sugar sources by volatile organic compounds (VOCs) released from the host plants and by some visual cues. The VOCs appear to be the dominant stimuli guiding mosquitoes from intermediate or long distances to the flowers of their plant hosts or to decaying fruit, whereas visual stimuli are principally the showy white or pale petals that can be detected only within a meter or two. The plants gain by releasing VOCs that serve as attractive signals to pollinators, making the vectors nectar thieves in most cases. It is not yet clear whether vectors are sometimes attracted to specialist-pollinated plants that have nectar inaccessible to vectors. Plant VOCs have not yet been used in surveillance, apart from incidental information gained while evaluating the attractiveness of potential host plants and crude fruit-based attractants (Müller and Schlein 2004,

2006, Müller *et al.* 2008, 2010a, Reisen *et al.* 1986, Schlein and Müller 2008). An effective field-tested synthetic odour blend, based on a plant's natural VOC headspace, remains to be developed.

The advantages of using phytochemical lures in traps for mosquitoes, and major hurdles that must be cleared, have been reviewed and described in detail in Foster and Hancock (1994) and Foster (2008). The main advantages are (1) attraction of both males and females of all ages of nearly all species of mosquitoes, (2) early detection, because sugar feeding is often the first activity after emergence, and males tend to emerge first, (3) localization of emergence sites, because males tend to remain more localized, (4) attraction of females in all gonotrophic states, not just those in the blood-seeking mode, and (5) attraction of females in reproductive diapause, when they will not seek blood. In other words, plant-volatile baited traps would target a much wider segment of a mosquito population than is typically sampled with CO_2-baited traps and ovitraps and would not be so seasonally restricted in temperate zones.

Because plant-sugar feeding usually occurs in the same general activity period as blood-host seeking, there is likely to be competition between the two resources, and it is generally thought that blood-host volatiles will be dominant over floral volatiles. However, during early life this may not be the case, and the relative strengths of the volatiles do matter. An appealing option is to combine phytochemicals with vertebrate kairomones, possibly gaining the advantages of each. But whether these volatiles would be additive, synergistic, or mutually inhibitory remains to be tested. The combination of phytochemicals with oviposition-site volatiles in gravid traps also might be effective (females may prefer to oviposit near sites where they and their offspring can quickly regain energy) and is worth considering.

The major hurdle is then simply the identification and synthesis of a plant-volatile blend that is attractive to mosquitoes at a high release rate so that it can out-compete naturally occurring plant odours. A complication is that relatively little is known about the attraction of mosquitoes to specific plant volatiles. An example of this is that the chemical cues coming from extrafloral nectaries of host plants and from host plants that must be pierced to obtain sugar, are almost completely unstudied. The latter clearly are attractive at a distance to the sand flies that feed on them (Junnila *et al.* 2010, Müller *et al.* 2011, Schlein and Müller 1995, Schlein and Yuval 1987). And Schlein and Müller (2008) reported that branches of similar plants with or without honeydew did not differ in the numbers of mosquitoes attracted, suggesting that honeydew itself is not attractive. Honeydew, though readily fed upon, appears not to be attractive to sand flies either (Müller and Schlein 2004, Müller *et al.* 2011). However, sand fly attraction to an aphid alarm pheromone has been demonstrated and may act as a cue to the presence of honeydew (Tesh *et al.* 1992).

Therefore, mosquitoes discover sugar by tarsal chemoreception when resting on plants, or they may be attracted to general plant volatiles and locate the nectaries by random walk. However, activation and orientation of mosquitoes towards floral VOCs are indisputable, and strong attraction to flowers of particular plant species has been shown. For instance, in a small bioassay chamber probing response of *Ae. aegypti* to extracts of milkweed (*Asclepias syriaca* L.) was greater than to extracts of Canada goldenrod (*Solidago canadensis* L.), the former being the more fragrant flower (Vargo and Foster, 1982). And there is upwind attraction of *Ae. aegypti* to isolated floral odours of ox-eye daisy (*Leucanthemum vulgare* Lam.), but an extract did not elicit landings (Jepson and Healy 1988). A role for floral odours in the mediation of upwind nectar-source location also was demonstrated by the attraction of *An. arabiensis* to an extract of *Achillea millefolium* L., a temperate plant. The major component of the odour was reported to be a cyclic

or bicyclic monoterpene (Healy and Jepson 1988). Mauer and Rowley (1999), using *Cx. pipiens*, confirmed that milkweed flowers are attractive in an olfactometer, but synthetic blends they created were not. By contrast, both individual components and synthetic blends of *Silene otites* (L.) Wibel headspace were found to be attractive to the *molestus* form of this species (Jhumur *et al.* 2006, 2007, 2008). Of 36 odour-receptor neurons on type A2 sensilla trichodea of female *Cx. pipiens*, 19 were relatively specific to bicyclic monoterpenes containing a ketone group (thujone and verbenone). The other 17 sensilla were more broadly tuned, and also were sensitive to other compounds, such as green-plant odours. Upwind flight was not elicited by exposure to each of these terpenes alone, or in combination with CO_2, suggesting that an odour blend is needed for plant location (Bowen 1992b). Carey *et al.* (2010) found that individual odourant receptors of *An. gambiae* that had strong responses to esters and aldehydes, volatiles common in the headspace of fruits, were all broadly tuned. Their role in discrimination between such volatiles is not entirely clear, but narrowly-tuned receptors often appear to be associated with salient, i.e. ecologically highly relevant, odours. A comprehensive study on the breadth of odourant sensitivity to volatiles present in attractive floral headspace has not yet been performed.

If mosquitoes have strong preferences for certain volatile blends, baits may be able to out-compete natural sources, unless nectar sources are very abundant. In recent years, more information on the specific attraction of certain plants and sugar sources has come from studies in Israel and Mali. A general impression is that there are certain super-attractants, and even natural concoctions based on these sources are effective in sugar baits. Their use, in combination with insecticides, is discussed below.

Salivation to detect pathogens

Mosquitoes and sand flies salivate while feeding on sugar, both to break down oligosaccharides with α-glucosidase – and perhaps also to break down starch with amylase – and to dilute very concentrated sugar solutions to facilitate ingestion. While salivating, they release viruses and malaria sporozoites (Beier *et al.* 1991, Billingsly *et al.* 1991, Russell *et al.* 1963, Van den Hurk 2007). Van den Hurk *et al.* (2007) tested whether this made it possible to determine infectivity rates of vectors without removing and testing salivary glands or testing extracts of whole insects. After mosquitoes were fed on a blood/virus mixture and provided with sucrose-soaked cotton pledgets after an extrinsic-cycle period, viral RNA was detected in the pledgets by RT-PCR. To see if this could be used as a convenient monitoring system, CO_2-baited updraft box traps were deployed in the field, in which mosquitoes would feed on honey-soaked cards that preserve nucleic acids (Hall-Mendelin *et al.* 2010*)*. These cards were collected once per week and presence of viruses successfully detected. The main advantage of this method over previous ones is its ease, because only the cards need to be tested for virus, which then can be associated with the species of mosquitoes within the trap, even after the mosquitoes have died and their viral contents corrupted. This method speeds up the turn-around time, improving early-warning systems.

Reduction of population density and age by deploying toxic sucrose solutions

Treatment of resting sites, including vegetation

When combined with a 20% sugar solution, Lea (1965) found that surface application of malathion, used as a residual insecticide, killed *Ae. aegypti* mosquitoes at one-tenth the dosage required otherwise. This effect was apparently because the irritancy of the mixture was offset by

its gustatory stimulation, so that mosquitoes remained in contact with it longer and perhaps also fed on it. The duration of its effectiveness also was extended considerably.

Over recent years, a control method that cleverly exploits sugar feeding has been developed and tested in several different environments with several species of mosquitoes. Attractive toxic sugar baits (ATSB) employ fruit scents to attract both male and female mosquitoes, a sucrose solution to stimulate feeding, and an oral insecticide – either boric acid or spinosad, both having very low vertebrate toxicity. Theoretically, the development of resistance can be avoided by rotating among many oral insecticides, although evolution of behavioural resistance (e.g. avoiding sugar sources) is a potential concern.

That this technique is effective in arid areas with relatively few flowering plants was demonstrated by spraying *Aristida raddiana* Savi (the only local flowering plants at the time) with dyed toxic sugar solution in a small oasis in Israel. In the control site, where the same solution was applied minus the toxin, between 80-90% of *An. sergentii* and 72-86% of *Ae. caspius* Pallas were marked with the dye, indicating that they rely predominantly on this plant species for their sugar. Both populations were eliminated in the treatment oasis (Müller and Schlein 2006). In another study, *Cx. pipiens* was shown to be strongly attracted to flowering *Tamarix jordanis* Boiss. branches. Treatment of just this one species with toxic sugar reduced *Cx. pipiens* numbers tenfold, although after 18 days the population rebounded (Schlein and Müller 2008). Similarly impressive results were obtained when the attractant was the juice of rotting nectarines and red wine, when sprayed on vegetation surrounding a sewage pond (Müller *et al.* 2010c). Further examples of the potential of this technique were demonstrated by applying boric acid in sugar solutions on the leaves and stems of vegetation in outdoor screen cages as well as smaller cages, which resulted in significant mortality of *Ae. albopictus* and *Cx. nigripalpus*, as well as a reduction in human landing rates. But *Aedes taeniorhynchus* Wiedemann was apparently unaffected by the boric acid solution (Xue *et al.* 2006). Even sub-lethal exposures reduced host seeking, fecundity, and survival of *Ae. albopictus* (Ali *et al.* 2006).

Perhaps the most relevant question pertains to the applicability of this method to *An. gambiae s.s.*, not only because it is one of the prime vectors of *falciparum* malaria in sub-Saharan Africa, but because the reliance of females on sugar in the field is a subject of debate. A field trial in Mali suggests we should be hopeful, because mosquito abundance dropped by 90% following the spraying of guava and honey-melon based ATSB on patches of vegetation of unknown attractiveness surrounding larval sites, and the percentage of females reaching at least 3 gonotrophic cycles dropped from 37 to 6% in the treatment area (Müller *et al.* 2010b).

Overall, these results suggests toxic sugar baits may be highly effective in semi-arid areas of Africa, especially where breeding sites are spatially segregated from domestic areas. The method is cheap and easy to implement, the attractant is easy to produce, and the approach can be used synergistically with ITNs.

Attractive toxic bait stations

As an alternative to spraying toxic sugar solution on vegetation, the use of sugar feeding stations also has been developed and tested. These consist of soda bottles filled with the solution (overripe nectarine juice, wine and sugar, and an oral insecticide), with a hole cut into them through which a wick keeps a sock wrapped around the bottle moist. A hood is placed atop this construction to shield it from the elements (Müller and Schlein 2008). In one study, these were placed at

the openings of cisterns, the resting and larval development sites of *An. claviger* Meigen. After introduction of the baits the number of females in that area decreased ten-fold (Müller and Schlein 2008). These bait stations also were used in a study in oases, this time suspended from *A. raddiana* trees. Baits that were laced with an insecticide steadily reduced the *An. sergentii* population to less than one tenth, and *Ae. caspius* to one third of the starting population (Müller *et al.* 2008). It is not immediately clear why the results, while impressive, were not as dramatic as the 2006 study, when full elimination was achieved. It may indicate that the bait stations are less effective than the spraying of resting vegetation.

Whether this station-based ATSB approach will work when they are placed in houses with vertebrate hosts, in lush areas with greater competition of natural nectar sources, or in urban areas where humans and breeding sites are closer together, remains to be seen. And, its impact on EIR or malaria prevalence has not been studied. A principal concern in the application of ATSB is its impact on non-target sugar feeding insect orders, such as Hymenoptera, Lepidoptera, Coleoptera, and Blattaria. This is unlikely to be an issue when applied within houses, where elimination of ants and cockroaches is welcome, and risk to humans is negligible. But outdoors, lethality to such beneficial insects as parasitoid wasps and various pollinating bees, moths, beetles, and other dipterans, requires special consideration. A partial way around this obstacle is to design attracticide stations that allow access to mosquitoes and other vectors but exclude large-bodied pollinators. An alternative is to provide a toxin whose action is specific to nematocerous dipterans or to the pathogens they carry.

Selective plant removal or replacement

A potential alternative use of plant feeding to control vectors, not involving insecticides, is the selective removal and replacement of their principal sources of sugar (Abdel-Malek and Baldwin 1961, Abdel-Malek 1964). In mesocosms, removal of sugar sources causes a dramatic reduction in *An. gambiae* reproduction, to the point of creating an environment that cannot sustain the population (Stone *et al.* 2009). To be practical and to have a minimal impact on the environment, this approach requires that the host plants be both few in diversity and low in density. Obviously, if a vector can use any of 5 or 10 different plant species in a transmission zone, and at least some of them are abundant, their removal and replacement would be onerous (Schaefer and Miura 1972), except close to human habitations. To identify opportunities where this approach is feasible, we first must overcome the obstacle of determining innate and learned plant-host breadth. Following removal of sources with highly attractive cues, will the ability of mosquitoes to efficiently locate nectar be significantly diminished, or will the mosquitoes simply shift to the next best plant?

One topic that will be important to plant-based control is the contribution of homopteran-generated honeydew to sugar meals of vectors. If honeydew is a large part of the sugar diet, plant removal to diminish sugar meals will require identifying the homopteran's plant hosts. The few studies that have attempted to quantify this suggest large differences between mosquito species (Burkett *et al.* 1999, Russell and Hunter 2002). This subject is understudied, and more data are needed to make generalizations about honeydew's importance according to vector species and locality.

A different tactic is suggested by the occurrence of plants that are naturally toxic to vectors but that are nevertheless attractive to them. The ornamental *Bougainvillea* has this effect on *Ph. papatasi*, and circumstantial evidence indicates that in its vicinity sand fly numbers are much lower than in other locations (Schlein *et al.* 2001). Toxic species, if planted around human

habitations, might provide sustained natural suppression of vector densities and lower survival, thereby compromising their vectorial capacity. Such plants have not yet been discovered that work against mosquitoes or other vectors.

Inoculation with microorganisms

Sugar baits have been used to induce *Cx. pipiens* mosquitoes to pick up the pathogenic bacterium *Bacillus sphaericus* and transfer it to larval development sites (Schlein and Pener 1990). Additionally, attention has been drawn to symbiotic gut bacteria that interfere with the ability of ingested mature *Plasmodium* gametocytes to form oocysts in the gut wall of *Anopheles* (Pumpuni *et al.* 1996). Attractive and palatable sugar baits have been tested as a means of spreading such bacteria into the mosquito populations (Lindh *et al.* 2006). Acetic-acid bacteria (Acetobacteraceae) are acquired naturally in nectars, fruit sugars, and phloem sap (e.g. Crotti *et al.* 2010, Suzuki *et al.* 2010, Yamada *et al.* 2000), invade most relevant organs of the mosquitoes' bodies, and are transmitted both horizontally and vertically within mosquito populations. Such bacteria show promise for generating anti-parasite molecules within vectors (Damiani *et al.* 2008, Favia *et al.* 2007, Riehle and Jacobs-Lorena 2005, Riehle *et al.* 2007). One candidate is the osmotolerant bacterium *Asaia*, strains of which can infect the major vector species of mosquitoes (Chouaia *et al.* 2010) and can be transformed easily with foreign DNA (Favia *et al.* 2007, 2008) to produce strains that inhibit malaria development, thereby eliminating its vector competence.

Conclusion

The aim of this review is to raise awareness of the potential effects vegetation abundance and composition may have on pathogen transmission dynamics. This overlooked aspect of the biology of mosquitoes shows tremendous promise for novel surveillance and control methods. Parallels between relevant sand fly and mosquito studies draw attention to gaps in the latter. Uneven knowledge of the topic and contradictory results currently frustrate our attempts to form a holistic view of the epidemiological consequences of sugar feeding in mosquitoes. Here, in summary, are four major questions in need of further research: (1) What is the nectar-host breadth of vector species? (2) What properties make certain plants attractive? (3) How is vector performance affected in the absence of highly attractive, nectar-rich plants? and (4) How do stimulus strength and perceived quality of plant and vertebrate volatiles interact to mediate host choice? Addressing these questions not only for anthropophilic species, but also for generalist and zoophilic mosquitoes, would broaden our current view of mosquito-nectar dynamics, which relies excessively on experimentation with *An. gambiae s.s.* and *Ae. aegypti*. Further, the use of natural systems would greatly improve our understanding of sugar's effect on vectorial capacity and reproductive success, by demanding more realistic energy expenditures. The best systems would include not only semi-field environments, but also wild-type mosquitoes, natural vector-parasite interactions, and natural blood hosts.

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4. Vector competence for arboviruses in relation to the larval environment of mosquitoes

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Abstract

Mosquito vector competence studies are reviewed to identify the species and environmental conditions that modify susceptibility to infection and transmission of pathogens. Most studies on vector competence have focused on environmental conditions experienced by adults, but the larval environment shapes the phenotypes of adult mosquitoes and may, thereby, alter vector competence for arthropod-borne (arbo)viruses. This review summarizes results of studies on the effects of nutrition, competition, temperature and insecticides during the larval stages on adult vector competence for arboviruses. A statistical analysis of previously published work supported the conclusion that larval environment may significantly alter susceptibility to infection, dissemination, and virus transmission. These effects show multiple and environmentally specific effects on barriers to virus infection. Involvement of multiple virus barriers in the adult mosquito suggests that several factors may be responsible for the alteration of vector competence by larval environment.

Keywords: arbovirus, competition, insecticide, nutrient limitation, temperature, vector competence

Introduction

Studies to the assess ability of mosquitoes to vector pathogens have played a central role in advancing our understanding of mosquito-borne disease transmission and led to improvements in control practices. Vector competence is the intrinsic permissiveness of an arthropod to infection, replication, and transmission of a pathogen (Hardy 1988, Woodring *et al.* 1996) and it serves as a measure of vectoring ability. Measurements of vector competence can be integrated with other ecological and behavioural characteristics of mosquitoes (abundance, longevity, feeding habit) to estimate vectorial capacity. Vectorial capacity, the average number of potentially infective bites received by a host in a single day, may be used to guide control efforts aimed at reducing contact rate between infected vectors and target hosts. A central goal of mosquito vector competence studies is to identify the conditions that modify competence in order to further our understanding of interactions between mosquitoes and pathogens and to aid efforts to minimize transmission risk. Both environmental and genetic factors influence arthropod-borne (arbo)virus infection processes in mosquitoes. Although numerous mosquito species may be capable of transmitting particular arboviruses, usually far fewer contribute substantially to transmission in nature because of genetic or environmental barriers related to vectoring ability, including vector competence. Most studies on vector competence have focused on environmental conditions experienced by adults (e.g. ambient temperature, viral dose) but environmental conditions of immature stages may have latent effects that continue to adulthood and alter arbovirus vector competence.

Growth and development of mosquitoes occurs during the aquatic stages, so the immature environment shapes traits of adults, some of which may relate to vector competence for arboviruses and disease transmission. The immature stages of mosquitoes occupy a variety of aquatic environments which vary in quality and quantity of nutrients, primarily from allochthonous detritus that forms the basal resources for microorganisms eaten by mosquito larvae (Fish and Carpenter 1982, Lounibos *et al.* 1993, Merritt *et al.* 1992, Walker *et al.* 1991, 1997). Different types

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and quantities of detritus influence individual development, growth, survival, as well as population dynamics and community structure (e.g. species assemblages), and these effects may be modified by ambient temperature. Equally as important are biotic interactions (e.g. predation, competition, parasitism), many of which primarily occur during the larval stages. Regulation of populations of mosquitoes in permanent ground water habitats is strongly influenced by predation, whereas competition appears to be a more important regulatory mechanism in ephemeral and container aquatic habitats (Juliano 2007, Service 1985).

Studies of mosquito vector competence, even when quantitative comparisons are made between species, rarely consider the possibility that biotic interactions may influence vector competence for arboviruses. For mosquitoes, biotic interactions shaping adult traits are largely experienced during the larval stages. Direct interactions include predation, interference, mutualism, and parasitism. Indirect interactions occur through intermediary species, and they can be most generally categorized as either feeding chains or other interactive modifications (Peacor and Werner 2000, 2001, Werner and Peacor 2003, Wooton 1994, 2002). Alterations in phenotypes from species interactions and other aspects of the larval environment are assumed to arise from phenotypic plasticity. Alternatively, alterations in phenotypes may also be due to selection among individuals for different phenotypes (Hetchel and Juliano 1997). Regardless of the mechanism, if alterations in phenotypes also translate to altered susceptibility to viral infection and transmission, then there is a possibility that the larval environment may influence disease transmission.

Typically, arbovirus transmission by mosquitoes involves acquisition in the midgut of an infectious blood meal, infection of midgut cells, dissemination of virus from the midgut to secondary tissues including the salivary glands, and inoculation of a vertebrate host during probing and feeding (Hardy *et al.* 1983, Woodring *et al.* 1996). Transmission from mosquito to host depends on whether the founding virus population, usually acquired by bite, successfully overcomes barriers to dissemination. The relative efficacy of barriers that impede the progression of virus infection in mosquitoes may depend on the specific virus, mosquito species, or even the geographic origin of the mosquito or virus (e.g. Bennett *et al.* 2002, Gubler *et al.* 1979, Tabachnick *et al.* 1985). Identified barriers to infection include the midgut infection and escape barriers and salivary gland infection and escape barriers (Hardy *et al.* 1983, Woodring *et al.* 1996). Both the midgut infection and escape barriers are dose-dependent so that higher doses of virus increase the probability of infection and its progression to other tissues. Differences in vector competence are reduced, sometimes to the point of eliminating resistance to viral infection in mosquitoes, when the midgut infection and escape barriers are bypassed via intrathoracic inoculation (Gubler and Rosen 1976, Hardy *et al.* 1978), underscoring the importance of natural infection processes to vector competence. The salivary gland infection and escape barriers ultimately determine whether a disseminated infection may be transmitted to a host through virus-infected saliva during probing and feeding.

Although our understanding of the molecular basis for barriers to virus infection is rudimentary, recent advances on the expression of immunity related genes have been possible by completion of annotated genome sequences for the mosquitoes *Aedes aegypti* (L.) and *Anopheles gambiae* Giles (reviewed in Fragkoudis *et al.* 2009). Ingestion of arboviruses is known to induce antimicrobial immune pathways including immune deficiency (Imd), Toll (Ramirez and Dimopoulos 2010, Sanders *et al.* 2005, Xi *et al.* 2008), Janus kinase-signal transducers and activator of transcription (JAK/STAT) (Fragkoudis *et al.* 2009, Sanders *et al.* 2005, Souza-Neto *et al.* 2009), and RNA interference (RNAi) (Cirimotich *et al.* 2009, Keene *et al.* 2004, Khoo *et al.* 2010). The relative role and sequence of immune signaling between linked pathways that lead to modification in viral infection and replication are only known in part for a few mosquito species and viruses *in vivo,* e.g. *Ae. aegypti*

and Sindbis virus (Cirimotich *et al.* 2009, Khoo *et al.* 2010, Sanders *et al.* 2005); and *Ae. aegypti* and dengue virus (Ramirez and Dimopoulos 2010, Sánchez-Vargas *et al.* 2009, Xi *et al.* 2008). Moreover, immune pathways may be virus-specific, as is the case with *Drosophila melanogaster* Meigen, and modified by the environment (reviewed in Xi *et al.* 2008). For instance, exposure to temperature and other types of environmental stress may up-regulate the expression of heat shock proteins in mosquitoes (Zhao *et al.* 2010). In some cases these proteins may influence virus infection, as demonstrated by *An. gambiae* heat shock protein cognate 70B which reduces o'nyong-nyong virus replication (Sim *et al.* 2007). If the environmental conditions experienced by larvae lead to expression of immune-related genes in nature, then such molecular changes may affect vector competence for arboviruses (Muturi, unpublished results).

There is an intellectual gap to be traversed between the ecology of the immature stages and adult vectoring ability. The current chapter limits our investigation into this area of research to vector competence for arboviruses. Our aim is to connect the importance of different environmental influences on larvae to mosquito biology and vector competence (Table 1). Specifically, we will use this review as a basis to formalize tests of hypotheses generated from published literature regarding the nature and extent of larval environmental influences on vector competence, identify plausible mechanism(s) for altered vector competence, and discuss areas for future research to further our understanding of larval environmental influences on susceptibility to virus infection and transmission. For purposes of this review, assays of mosquitoes for virus infection are categorized into infection, dissemination and transmission. Our reasoning is that these categories enable us to better identify particular virus barriers that may be modified by the larval environment. Infection refers to the presence of virus in mosquito midguts or in mosquito bodies and absence of virus in other tissues (e.g. legs). Dissemination refers to the presence of virus in tissues other than the midgut. Evidence for transmission comes either from the presence of virus in mosquito saliva or in vertebrate hosts after infected mosquitoes are allowed to probe/feed.

Nutrition

Decomposing organic matter in the form of plant detritus and decomposing invertebrates serve as the basal resources for microorganisms that form the diet for mosquito larvae (Kaufmann *et al.* 2010, Merritt *et al.* 1992). Spatio-temporal variation in the availability of organic matter is likely to determine habitat quality for mosquitoes. The quantity and quality of nutrients available to mosquitoes largely determine growth and development during the larval stages. Upon eclosion, the size and nutrient reserves of adults are directly related to the nutrient conditions that the larvae experienced. It has been long suspected that mosquito size, and associated physiological status and fitness, may influence susceptibility to virus infection and transmission. Studies on the role of larval nutrition on vector competence are biased towards container-inhabiting mosquitoes due to their importance in the transmission of arboviruses as well as their ease of manipulation.

Large adult *Aedes triseriatus* Say from nutrient rich larval conditions were less likely to be infected and transmit LaCrosse encephalitis virus (LACV) than small adults from nutrient-deprived larvae (Grimstad and Haramis 1984, Grimstad and Walker 1991, Patrican and DeFoliart 1985). These nutrient-dependent effects on transmission were observed for both horizontal (by bite) and vertical (transovarial) routes of transmission (Patrican and DeFoliart 1985). Although mechanism(s) responsible for altered vector competence were not identified, differences in the midgut morphology were suggested to be responsible for higher rates of LACV dissemination from the midgut in small-sized adults due to a thinner basement membrane (fewer basal laminae) than detected in large-sized adults (Grimstad and Walker 1991). Field collections of pupae were

Table 1. Larval environments of mosquitoes and adult vector competence for arboviruses.

Mosquito species	Arbovirus	Larval environment	Altered performance	Infection[1]	Dissemination[1]	Transmission[1]	Reference
Ae. aegypti							
	CHIKV	elevated temperature	development, survivorship	.	+	.	Mourya *et al.* 2004
	DENV-2	elevated temperature	.	.	+	.	Yadav *et al.* 2005
		intra-, interspecific competition	size, survivorship, development, λ′	0	0	.	Alto *et al.* 2008a
			size	+	+	.	Alto *et al.* 2008b
		intraspecific competition	size	.	-	.	Sumanochitrapon *et al.* 1998
	RRV	nutrient deprivation	size	-	.	.	Nasci and Mitchell 1994
	SINV	elevated temperature	size, development, survivorship, λ′	+	+	.	Muturi, unpublished results
			size, development, survivorship	+	+	.	Muturi and Alto 2011
		insecticide malathion	size, development, survivorship, λ′	+	+	.	Muturi and Alto 2011; Muturi, unpublished results
			size, development, survivorship	0	+	.	Muturi *et al.* 2011
		intra-, interspecific competition	size, survivorship, development, λ′	0	0	.	Alto *et al.* 2005
		intraspecific competition	size, survivorship, development	0	+	.	Muturi *et al.* 2011
		nutrient deprivation	size, development, survivorship	+	+	.	Muturi, unpublished results
Ae. albopictus							
	CHIKV	elevated temperature	size, development, survivorship	-	-	.	Westbrook *et al.* 2010
	DENV-2	intra-, interspecific competition	size, survivorship, development, λ′	+	+	.	Alto *et al.* 2008a
			size	+	+	.	Alto *et al.* 2008b
		nutrient deprivation	.	.	+	.	Zhang *et al.* 1993
	SINV	insecticide malathion	size, development, survivorship	0	0	.	Muturi *et al.* 2011
		intra-, interspecific competition	size, survivorship, development, λ′	+	+	.	Alto *et al.* 2005
		intraspecific competition	size, development, survivorship	0	0	.	Muturi *et al.* 2011
Ae. taeniorhynchus							
	RVFV	elevated temperature	.	-	+	.	Turell 1993
	VEEV	elevated temperature	.	-	+	.	Turell 1993

Table 1. Continued.

Mosquito species Arbovirus	Larval environment	Altered performance	Infection[1]	Dissemination[1]	Transmission[1]	Reference
Ae. triseriatus						
LACV	field collected pupae	size	.	+	+	Paulson and Hawley 1991
	intra-, interspecific competition	size, survivorship	+	+	.	Bevins 2008
	nutrient deprivation	size	0	+	+	Grimstad and Haramis 1984
		size	+	+	+	Grimstad and Walker 1991
		size	.	+	+	Paulson and Hawley 1991
		size, development	.	+	+	Patrican and DeFoliart 1985
Ae. vigilax						
RRV	elevated temperature	.	0	0	.	Kay and Jennings 2002
	nutrient deprivation	size	0	0	0	Jennings and Kay 1999
Cx. annulirostris						
MVEV	elevated temperature	.	+	.	+	Kay *et al.* 1989b
	nutrient deprivation	size	0	.	0	Kay *et al.* 1989a
Cx. tarsalis						
WEEV	elevated temperature	.	_	.	.	Hardy *et al.* 1990
Cx. tritaeniorhynchus						
JEV	nutrient deprivation	.	.	.	+	Takahashi 1976
WNV	elevated temperature	size, development, survivorship	0	.	.	Baqar *et al.* 1980
	intraspecific competition	size, development, survivorship	+[2]	.	.	Baqar *et al.* 1980
	nutrient deprivation	size, development, survivorship	0	.	.	Baqar *et al.* 1980

[1] Symbols +, -, and 0, show increases, decreases, and no change in infection parameters. Periods (.) indicate that these measurements were not recorded.
[2] Baqar *et al.* (1980) showed that *Culex tritaeniorhynchus* from intermediate larval rearing densities (2.0 larvae/ml) had significantly lowered rates of West Nile virus infection than mosquitoes from low (0.5 larvae/ml) and high (4.0 larvae/ml) densities.

used to further address whether the size of *Ae. triseriatus* in natural populations could be related to vector competence. Rates of disseminated infections and transmission after imbibing LACV infected blood were negatively related to mosquito size, as measured by both pupal weight and wing length for two strains of *Ae. triseriatus* (Paulson and Hawley 1991). These relationships appear to be attributable to environment and not genetic determinants (Anderson *et al.* 2005), since vector competence was indistinguishable among F1 progeny from well-fed larvae of small and large sized parental mosquitoes (Paulson and Hawley 1991). Similarly, small adult *Aedes albopictus* Skuse derived from nutrient-deprived larvae had significantly higher rates of dissemination of dengue-2 virus than large adults from nutrient rich larval conditions (Zhang *et al.* 1993). Electron microscopic observation of mesenteronal tissue showed that adults from nutrient-deprived larvae had thinner basement membranes (6-12 laminae) than adults from nutrient rich larval conditions (14-19 laminae). Moreover, adults from field collections of *Ae. albopictus* pupae had body sizes and rates of dengue-2 virus infection similar to those adults from nutrient-deprived larval conditions (Zhang *et al.* 1993). Takahashi (1976) showed a trend for reductions in the length of the extrinsic incubation period for Japanese encephalitits virus in *Culex tritaeniorhynchus* Giles from nutritionally-deprived larvae. However, the relationship between nutrition and vector competence for other mosquito species and viruses has yielded inconsistent outcomes ranging from no effects, e.g. *Aedes vigilax* Skuse and Ross River virus (Jennings and Kay 1999); *Culex annulirostris* Skuse and Murray Valley encephalitis virus (Kay *et al.* 1989a), to decreasing competence with nutrient deprivation, e.g. *Ae. aegypti* and Ross River virus (Nasci and Mitchell 1994). Thus, nutrient-dependent changes in competency for arboviruses may, at least in part, depend on the species of mosquitoes and viruses.

Intra- and interspecific competition

Density-dependent interactions play a critical role in regulating populations and may have implications for understanding vector biology, control, and improved predictability for risk of disease transmission. For mosquitoes these regulatory forces act strongly on the immature aquatic stages and subsequently impact the size and fitness of the adult population. Regulation of populations of mosquitoes using container and ephemeral habitats appears to be strongly influenced by density-dependent competition, a source of mortality (e.g. Arrivillaga and Barrera 2004, Barrera and Medialdea 1996, Barrera *et al.* 2006, Juliano 2007, Service 1985, Southwood *et al.* 1972). Along the same lines, management of mosquito-borne diseases largely depends on the application of larvicides that induce mortality and subsequently reduce the density of adult mosquito vectors. Surprisingly, we know very little in terms of how these multiple sources of mortality influence arboviral vector competence of surviving adults.

One of the earliest studies to investigate the relationship between competition and vector competence focused on *Cx. tritaeniorhynchus* and infection with West Nile virus (Baqar *et al.* 1980). Moderate competition (2 larvae/ml) significantly reduced susceptibility of *Cx. tritaeniorhynchus* to West Nile virus infection relative to low and high competition (0.5 larvae/ml and 4 larvae/ml, respectively). Although a clear pattern of larval density effects on infection with West Nile virus was not identified, the study showed that the larval environment has effects that continue to influence interactions between the adult mosquito and arboviruses. Nearly two decades later a competition experiment using F1 *Ae. aegypti* from field collections in three geographic locations in Thailand demonstrated that large mosquitoes from low intraspecific competition had higher dissemination of dengue-2 virus than small mosquitoes from high competition (Sumanochitrapon *et al.* 1998). These effects also appeared to be stronger in mosquitoes from some geographic

locations over others, suggesting a possible interaction between genotype and competition in dengue vector competence.

Only recently have researchers begun to investigate the role of interspecific competition in modifying arbovirus vector competence in mosquitoes. Research in this area was prompted by widespread invasions by the Asian tiger mosquito *Ae. albopictus* (reviewed in Benedict *et al.* 2007). *Aedes albopictus* immature stages occupy water-filled containers along with other mosquito species. In the USA, the treehole mosquito *Ae. triseriatus* and yellow fever mosquito *Ae. aegypti* are common occupants of containers and vectors of LaCrosse and dengue viruses, respectively. Competitive interactions between these species are well documented and influence individual life history traits, population dynamics and community structure. The establishment of *Ae. albopictus*, a competent laboratory and natural vector of several arboviruses, in new regions poses a risk to human health because it may become involved in existing disease transmission cycles of established pathogens or newly introduced pathogens. Additionally, interspecific interactions between *Ae. albopictus* and other mosquito species may alter the relative abundance of the adult populations of vector species or affect the phenotypes of mosquitoes that relate to vectoring ability such as vector competence.

Ae. albopictus is competitively superior in its larval stages to native *Ae. triseriatus* (Aliabadi and Juliano 2002, Livdahl and Willey 1991, Novak *et al.* 1993, Teng and Apperson 2000). Therefore, interspecific interactions between the larvae of these species are predicted to impose asymmetric nutritional stress on *Ae. triseriatus* via resource depletion, perhaps with similar consequences for susceptibility to LACV infection as was the case among nutritionally-deprived *Ae. triseriatus* (see above). Using a replacement series design, Bevins (2008) investigated competitive interactions between larvae of *Ae. triseriatus* and *Ae. albopictus* and their consequences for *Ae. triseriatus* infection with LACV. The presence of *Ae. albopictus* increased *Ae. triseriatus* mortality but the surviving individuals were larger than in treatments containing only *Ae. triseriatus*, perhaps attributable to release from competition among survivors. Reciprocal effects on *Ae. albopictus* performance measurements were not observed, consistent with other studies that demonstrated the competitive superiority of *Ae. albopictus*. Large *Ae. triseriatus* from interspecific treatments (containing *Ae. albopictus*) had higher infection and dissemination rates of LACV compared to intraspecific treatments of *Ae. triseriatus* (Bevins 2008). In contrast to studies on nutrition and LACV infection in *Ae. triseriatus* (see above), larger, not smaller, mosquitoes had enhanced vector competence, suggesting that size alone is not an accurate predictor of susceptibility to infection and transmission of LACV. Despite the apparent release from competition for *Ae. triseriatus* in interspecific treatments, the viral competency of these large sized individuals was still enhanced due to interactions with *Ae. albopictus*, a superior competitor. Studies on competitive interactions in mosquitoes commonly measure multiple population growth correlates as well as an estimate of the per capita rate of change (e.g. r', Livdahl and Sugihara 1984) because in many instances the effects of competition are complex, and interpretation of single or a few performance measurements may not accurately reflect outcomes at the population level. It is therefore plausible that the large *Ae. triseriatus* with enhanced competence for LACV from interspecific treatments experienced stress in other ways that were not fully captured by measuring size-related effects of competition.

The invasion of *Ae. albopictus* in the USA has been well-documented, including its influence on established mosquito species (Moore 1999, O'Meara *et al.* 1995, 1993). The spread of *Ae. albopictus* in southeastern USA in the 1980-1990s was associated with a decline in the abundance and geographic distribution of the yellow fever mosquito *Ae. aegypti* (Hobbs *et al.* 1991, Mekuria and

Hyatt 1995, O'Meara *et al.* 1995). Interspecific interactions between these two species appear to have contributed to declines in *Ae. aegypti* (Juliano *et al.* 2004). Several mechanisms have been proposed to explain displacements of *Ae. aegypti* populations (reviewed by Lounibos 2002, 2007), but superiority of *Ae. albopictus* in larval resource competition has received the most attention (e.g. Juliano 1998). Using a small response surface design, Alto *et al.* (2008a) showed intraspecific and interspecific larval competition altered individual life history traits, population performance, and vector competence for dengue-2 virus (DENV-2). Specifically, for laboratory strains of *Ae. albopictus,* but not *Ae. aegypti,* high levels of competition enhanced susceptibility to infection and dissemination with DENV-2, and these effects were presumed to be attributable to reductions in midgut infection and escape barriers (Figure 1) (Alto *et al.* 2008a). In a companion study, smaller *Ae. aegypti* and *Ae. albopictus* were more likely to become infected and to disseminate DENV-2 than larger individuals, independent of rearing conditions (Alto *et al.* 2008b). In a similarly designed experiment completed earlier using the same mosquito strains, competition-enhanced infection in *Ae. albopictus* with Sindbis virus (SINV) was observed (Alto *et al.* 2005). A separate study using more recently colonized Florida strains of mosquitoes showed that intraspecific competition enhanced dissemination of SINV in *Ae. aegypti* but not *Ae. albopictus* (Muturi *et al.* 2011). Although reasons for differences in the results between species and strains remain unclear, these studies showed a general relationship between competitive stress (lengthened development, reduced growth and survival) and weakening of midgut barriers. Also, it appears that competitive stress may alter other aspects of viral infection because high competition significantly increased SINV, but not DENV-2, viral titer in *Ae. albopictus* with disseminated infections, suggesting a reduction in the innate immune response to limit virus replication (Alto *et al.* 2005). Although the mechanism(s) responsible for these observed effects were unclear, it appears that multiple immune processes may be compromised because midgut barriers and viral titer were modified by the effects of

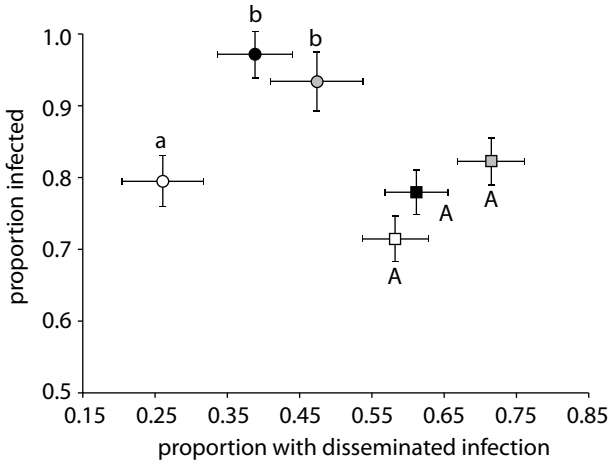

Figure 1. Proportion of Aedes aegypti *and* Aedes albopictus *infected and with disseminated infections after fed blood containing dengue-2 virus. Mosquitoes were exposed to intra- and inter-specific competition as larvae (Alto* et al. *2008a). Competition treatments consisted of initial number of larvae of* Ae. albopictus *:* Ae. aegypti *per container – 160:0 (open circle), 320:0 (filled circle), 160:160 (grey circle and grey square), 0:320 (filled square) and 0:160 (open square). Bivariate means followed by different lower- and uppercase letters show significant differences for* Ae. albopictus *and* Ae. aegypti, *respectively. Circles show* Ae. albopictus *and squares show* Ae. aegypti.

competition. Differences in competitive effects on virus replication may be related to the type of arbovirus being considered (e.g. SINV = *Alphavirus*, DENV = *Flavivirus*) as well as the species and strain of mosquito.

Temperature

Temperature is one of the most commonly studied of environmental factors affecting biological processes, including seasonal and geographic differences in temperature as well as anticipated climate change effects. Understanding temperature impacts on interactions between arthropods and pathogens will assist in predicting disease transmission patterns which are likely to be altered by anticipated climate change (Lafferty 2009, Rogers and Randolph 2006, Tabachnick 2010). The body temperature of mosquitoes is directly proportional to ambient temperature, thereby influencing growth, development, population dynamics (Alto and Juliano 2001a,b, Lounibos *et al.* 2002) and arbovirus infection including the extrinsic incubation period (EIP) which is the time from initial acquisition of virus infection to capacity to transmit (Chamberlain and Sudia 1955, Hardy *et al.* 1983). Consistent observations have documented that increases in adult maintenance temperatures are associated with enhanced vector competence as measured by higher net rates of infection, transmission and reductions in EIP for several viruses and mosquito species (e.g. Anderson *et al.* 2010, Chamberlain and Sudia 1955, Davis 1932, Hardy *et al.* 1983, Kay and Jennings 2002, Kay *et al.* 1989a,b, Richards *et al.* 2009, 2007, Turell 1993).

Although most investigations of temperature effects on vector competence have focused on adult mosquitoes, fewer studies have determined whether ambient temperatures acting on the immature stages of mosquitoes affect the vector competence of adults for arboviruses. However, temperatures experienced during the immature stages shape the adult phenotype e.g. nutritional reserves (Briegel and Timmermann 2001, Briegel *et al.* 2001a,b), so it seems plausible that such temperature effects also modify interactions between arboviruses and mosquito adults. *Aedes taeniorhynchus* Wiedemann reared at 19 °C had significantly higher infection, but not dissemination, rates of Rift Valley fever and Venezuelan equine encephalitis viruses than mosquitoes from 26 °C (Turell 1993). Larval rearing temperatures of 18, 24, and 32 °C altered development and growth rates of *Ae. albopictus* and resulted in adults with different competence for chikungunya virus. Infection rates did not differ among mosquitoes, but *Ae. albopictus* from 18 °C were significantly larger and had dissemination rates six times higher than mosquitoes from the warmest larval rearing conditions (32 °C) (Westbrook *et al.* 2010, Figure 2). These results suggest that the global spread of CHIKV vector *Ae. albopictus* in recent decades (Juliano and Lounibos 2005, Lounibos 2002) coupled with emergence of chikungunya virus in Italy (Powers and Logue 2007, Rezza *et al.* 2007), La Réunion (Paquet *et al.* 2006), Indonesia, Sri Lanka, and Singapore (Seneviratne *et al.* 2007) may pose greater epidemiological risks. In particular, high altitudes such as highlands of La Réunion where *Ae. albopictus* develops in cooler temperatures, may enhance risks of disease transmission. However, we caution against firm conclusions at this point since temperature is known to alter other parameters of vectorial capacity, and the net effect on risk of disease transmission is unclear. Results from this study are consistent with other field studies where cooler temperatures correlate with enhanced vector competence of Western equine encephalitis virus in a population of *Culex tarsalis* Coquillett (Hardy *et al.* 1990) and Murray Valley encephalitits virus transmission by *Cx. annulirostris* (Kay *et al.* 1989b). Taken together, these studies suggest that temperature effects experienced during the immature stages have opposite effects on arboviral competence compared to incubation temperature of adults (i.e. positive associations between temperature and vector competence). Low rearing temperature of immature stages typically results in larger sized adult mosquitoes (e.g. Lyimo *et al.* 1992; Westbrook *et al.* 2010) that imbibe larger volumes of blood and

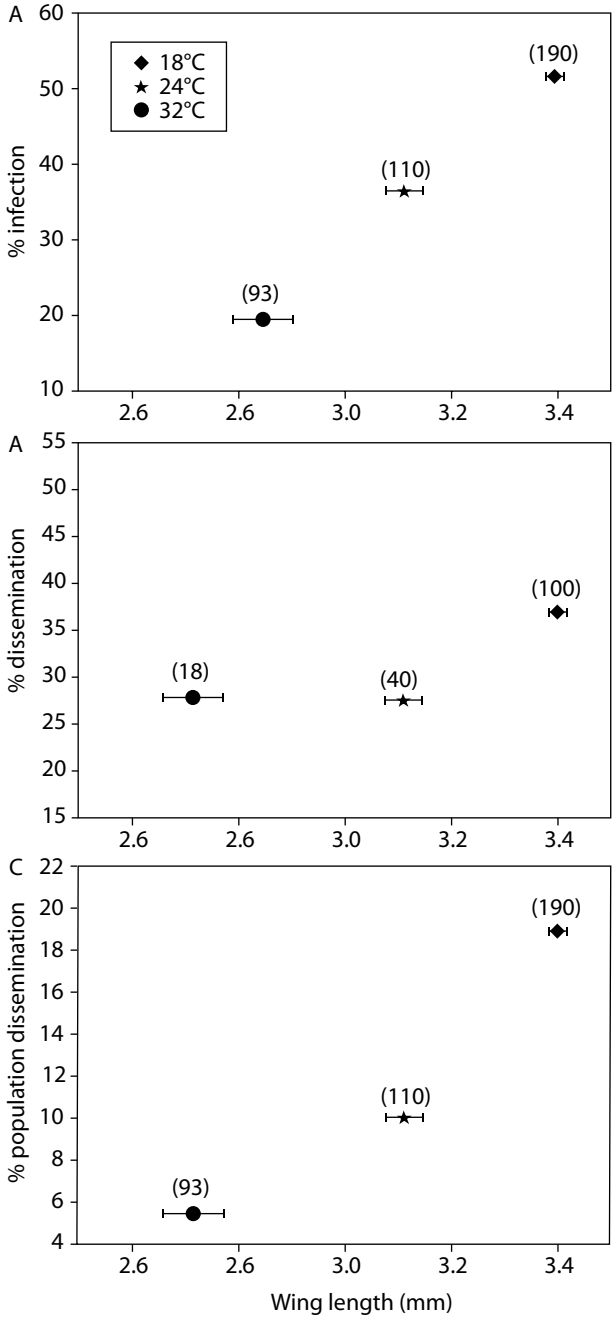

Figure 2. Percent of Aedes albopictus *infected and with disseminated infections after fed blood containing chikungunya virus. Mosquitoes were exposed to different temperatures as larvae. Numbers in the parentheses show the number of mosquitoes assayed for viral infection (Westbrook* et al. *2010).*

presumably more virus. It is therefore plausible that higher initial doses of viruses imbibed by large mosquitoes reared at cool temperatures may play a role in altered vector competence.

Other studies have found little or no identifiable relationships (Baqar *et al.* 1980, Kay and Jennings 2002) or opposite effects between larval rearing temperature and viral competence in mosquitoes. Elevated rearing temperature (30 vs. 20 °C or 32 vs. 25 °C) during larval and pupal development led to enhanced rates of infection and dissemination with adult *Ae. aegypti* and Sindbis virus (Muturi and Alto 2011, Muturi, unpublished results). Similarly, short-term exposure of larvae to heat shock (range from 36-44.5 °C) enhanced vector competence of *Ae. aegypti* for chikungunya virus (Mourya *et al.* 2004) and DENV-2 (Yadav *et al.* 2005). The molecular basis for observed temperature-dependent enhancement or depression of infection or dissemination is not well studied, but some researchers have hypothesized that temperature-induced up-regulation and down-regulation of stress (e.g. heat shock proteins) and immunoresponsive genes may affect susceptibility of mosquitoes to viral infection (Mourya *et al.* 2004, Muturi, unpublished results, Yadav *et al.* 2005).

Insecticides

Integrated mosquito management heavily relies on the use of insecticides directed at adult and immature mosquitoes. Control efforts assume that an externally applied source of mortality (e.g. insecticide) will act additively with other sources of mortality in nature to reduce overall numbers of adult mosquitoes. However, there may be environmental conditions that promote alternative outcomes, a possibility when two or more sources of mortality act in non-additive ways to reduce or increase the numbers of adult mosquitoes (Juliano 2007, Service 1985). For example, simulated mortality through removal of larvae in water-filled containers with suboptimal nutrients increased the number and size of *Ae. aegypti* that emerged to adulthood (Agudelo-Silva and Spielman 1984). If these results hold true under natural conditions then mosquito control by larviciding in nutrient-limited populations may increase the number of adults and perhaps life span of adults due to evidence for positive relationships between size and life expectancy in *Aedes* mosquitoes (Briegel *et al.* 2001, Haramis 1985, Reiskind and Lounibos 2009, Steinwascher 1982). Furthermore, insecticide-induced alterations in surviving mosquitoes may be associated with phenotypic variations in arboviral vector competence, either through sublethal exposure of insecticides or indirectly by modifications in the environment (e.g. release from competition). For example, insecticide resistant genes may in some instances have pleiotropic effects and result in changes in vector ability such as longevity, behaviour, and arboviral vector competence (reviewed in Rivero *et al.* 2010). Phenotypic variation related to mosquito vectoring ability could be due to a plasticity which would occur in the life span of the mosquito or selection where an alteration in the genetic structure of the population has occurred across generations (e.g. insecticide resistance).

To examine the interaction between competition and insecticide treatment, Muturi *et al.* (2011a) exposed Florida strains of *Ae. aegypti* and *Ae. albopictus* to intraspecific larval competition and a low concentration of the organophosphate insecticide malathion. They tested the hypothesis that the presence of malathion would alleviate larval competition and alter vector competence for Sindbis virus. This investigation, as well as follow-up studies, deliberately focused on container-inhabiting *Aedes* because the effects of competition are known to affect their vector competence and regulation of populations. As predicted, competition and malathion treatments reduced survival to adulthood. The presence of malathion appeared to have reduced competition as demonstrated by faster development and larger mosquitoes among survivors. However, it is also possible, but perhaps less likely, that malathion selectively favoured larger and faster developing mosquitoes. Exposure to malathion and competition independently led to a doubling of the

rate of virus dissemination in *Ae. aegypti* but not *Ae. albopictus*. Enhanced viral competence was observed among small, competitively stressed mosquitoes and large individuals exposed to malathion, an indication that size alone was not responsible for altered competence. These effects of malathion on vector competence suggest a degree of complexity that is not simply attributable to an alleviation of larval competition. If this were the case, then we would expect that *Ae. aegypti* exposed to malathion should have similar competence as *Ae. aegypti* in low competition intraspecific treatments in the absence of malathion. On the contrary, malathion-exposed *Ae. aegypti* had elevated rates of dissemination, similar in magnitude to those observed for *Ae. aegypti* from high larval competition treatments in the absence of malathion. Therefore it seems that direct exposure to malathion, and not indirect effects of malathion-mediated release from competition, alters arboviral vector competence, perhaps attributable to differential expression of immunity related genes induced by the presence of malathion. Additionally, the effects of malathion on viral competence may be modified by other environmental factors. For example, exposure to malathion during development of the immature stages at high temperature (30 °C) enhanced *Ae. aegypti* viral infection and dissemination with Sindbis virus, but these effects were not observed at low temperature (20 °C) (Muturi and Alto 2011). This latter study reaffirmed the notion that size *per se* does not dictate vector competence since the sizes of mosquitoes with enhanced competence in the presence and absence of malathion at 30 °C were similar (Muturi and Alto 2011).

Muturi (unpublished results) exposed Florida strains of *Ae. aegypti* to one of several environmental stressors during the aquatic stages (starvation, nutrient limitation, insecticide malathion or elevated temperature) to identify differential expression of stress and immunity-related genes and to relate these to vector competence for Sindbis virus. Differential expression of stress and immunity-related genes were measured in larvae and adult female *Ae. aegypti*. In larvae, elevated temperature was associated with up regulation in gene expression of cecropin, defensin and CYP6Z6, whereas suboptimal nutrients and exposure to a low dose of malathion increased expression of cecropin and transferrin, respectively, relative to the controls. In contrast, starvation led to down-regulation of defensin, cecropin, transferrin, HSP70, HSP83, and CYP6Z6. A subset of these genes was investigated in adult females. Transferrin was up-regulated in all treatments except starvation, and defensin was up-regulated in starvation and the elevated temperature treatments. Variation in mosquito performance (survival, development, size) was specific to the particular environmental stressor, but all stressors enhanced susceptibility to viral infection and dissemination among infected mosquitoes. Although the specific details of immune pathways still require resolution, these observations suggest that environmental conditions experienced by the immature stages may modify the expression of genes related to viral infection in mosquitoes.

Synthesis of environmental influences on vector competence

Our review of the literature suggested that environmental influences on larvae have, in some instances, consequences for vector competence for arboviruses. Further, the relationship between larval environment and vector competence may differ depending on the particular environment. In order to provide additional resolution on the issue, we extracted information on sample sizes and susceptibility to infection, dissemination, and transmission from all the studies presented in Table 1 to construct tests for the following null hypotheses:

Null hypotheses:

H_0: Nutrient deprivation does not alter vector competence
H_0: Competition does not alter vector competence
H_0: Elevated temperature does not alter vector competence
H_0: Exposure to insecticide does not alter vector competence

Hypotheses were tested by maximum likelihood categorical analyses of contingency tables (PROC CATMOD, SAS 2002) based on counts of individual mosquitoes being categorized as + or – for the presence or absence of virus, respectively. That is, responses from individual mosquitoes were used as data points. Separate maximum likelihood (ML) ANOVA tests were used for each larval environment and measure of vector competence (infection, dissemination, transmission) in order to more clearly identify viral barriers that may be modified by the environment. In instances where more than two levels were available within larval environmental treatments we used only the extremes (e.g. low, high) and excluded intermediate levels. The goal of these tests was to identify overall patterns of larval environment and vector competence for arboviruses, so no attempt was made to separate analyses by virus or mosquito species.

A total of 6,377 mosquitoes was used for the analysis for susceptibility to infection, 7,073 mosquitoes for the analysis of disseminated infection and 1,665 for the analysis of virus transmission. The ML ANOVA showed no significant nutrition effect for susceptibility to infection with arboviruses. However, mosquitoes from nutrient rich conditions were less likely to disseminate and transmit virus than individuals from nutrient-deprived conditions (Table 2). Thus, larval nutrition appears to have negligible effects on midgut infection barrier and primarily alters vector competence through changes in the midgut escape and transmission barriers. The ML ANOVA demonstrated a significant competition effect for susceptibility to infection and virus dissemination with enhanced susceptibility to infection and dissemination in adults from high competition larval environments (Table 2). These results suggest that both midgut infection and escape barriers are compromised by larval competition. An assessment for competitive effects on virus transmission was not feasible since no transmission studies have been performed. The ML ANOVA showed that warm rearing temperature significantly decreased susceptibility to infection and virus transmission but did not significantly influence dissemination in infected mosquitoes (Table 2). Rearing temperatures appear to influence both midgut and virus transmission barriers. However, we caution the interpretation of the generality of temperature effects on virus transmission because it was based on a single study. The ML ANOVA demonstrated that exposure to low concentrations of insecticide enhanced rates of infection and dissemination (Table 2). Insecticidal exposure effects on virus transmission have not been performed to date.

An overall assessment of these results suggest that larval environment (1) does alter vector competence for arboviruses, (2) influences multiple virus barriers in the adult mosquito, and (3) appears to be directionally similar in its influence on virus barriers for a given larval environment (e.g. midgut infection and escape barriers for competition).

Table 2. Maximum likelihood analyses of variance for the effects of larval nutrition, competition, temperature and insecticide on susceptibility to infection, dissemination and transmission of arboviruses.

Source Vector competence measure	Direction of test[1]	Estimate	Std. Error	df	χ^2	*P*-value	References
Nutrition							
Infection	High, +	-0.0286	0.0314	1	0.83	0.36	Baqar *et al.* 1980, Grimstad and Haramis 1984, Grimstad and Walker 1991, Jennings and Kay 1999, Kay *et al.* 1989a, Muturi unplished results, Nasci and Mitchell 1994
Dissemination	High, +	-0.1767	0.0246	1	51.74	<0.0001	Grimstad and Haramis 1984, Grimstad and Walker 1991, Jennings and Kay 1999, Muturi unpublished results, Patrican and DeFoliart 1985, Paulson and Hawley 1991, Zhang *et al.* 1993
Transmission	High, +	-0.1736	0.0261	1	44.31	<0.0001	Grimstad and Haramis 1984, Grimstad and Walker 1991, Jennings and Kay 1999, Kay *et al.* 1989a, Patrican and DeFoliart 1985, Paulson and Hawley 1991, Takahashi 1976
Competition							
Infection	High, +	0.1140	0.0257	1	19.66	<0.0001	Alto *et al.* 2005, 2008a, Baqar *et al.* 1980, Bevins 2008, Muturi *et al.* 2011
Dissemination	High, +	0.1934	0.0234	1	68.08	<0.0001	Alto *et al.* 2005, 2008a, Bevins 2008, Muturi *et al.* 2011, Sumanochitrapon *et al.* 1998
Transmission[2]	.	.	.	.	.	.	.
Temperature							
Infection	High, +	-0.1505	0.0219	1	47.05	<0.0001	Baqar *et al.* 1980, Hardy *et al.* 1990, Kay and Jennings 2002, Kay *et al.* 1989b, Muturi and Alto 2011, Muturi unpublished results, Westbrook *et al.* 2010
Dissemination	High, +	0.0267	0.0238	1	1.26	0.2624	Kay and Jennings 2002, Mourya *et al.* 2004, Muturi and Alto 2011, Muturi unpublished results, Turell 1993, Westbrook *et al.* 2010, Yadav *et al.* 2005
Transmission	High, +	-0.2652	0.1265	1	4.39	0.0361	Kay *et al.* 1989b
Insecticide							
Infection	Control, +	-0.0713	0.0283	1	6.34	0.0118	Muturi and Alto 2011, Muturi *et al.* 2011, Muturi unpublished results
Dissemination	Control, +	-0.0966	0.0316	1	9.33	0.0023	Muturi and Alto 2011, Muturi *et al.* 2011, Muturi unpublished results
Transmission[2]	.	.	.	.	.	.	.

[1] Symbols + and – denote the presence or absence of virus. 'High' refers to the level of nutrients, competition and temperature. The 'direction of the test' denotes the reference point from which the test generates maximum likelihood (ML) estimates. Take for example nutrition effects on virus dissemination; there is a significant negative relationship (ML estimate of -0.1767) between high nutrition and disseminated virus infection.

[2] Periods (.) indicate that data are lacking to construct a statistical test.

Plausible mechanisms

The mechanisms responsible for changes in adult vector competence for arboviruses attributable to the larval environment are not entirely clear. In most instances published studies aimed to identify the nature of larval environmental effects on adult competence for viruses but not the mechanism(s) responsible. It seems reasonable to postulate that the mechanism(s) responsible for changes in adult competence attributable to nutrient limitation and competition may be similar, given that both these factors deprive larvae of nutrients and induce similar alterations in life histories (delayed development, reduced growth and survival). However, determinants of adult competence attributable to ambient temperature and exposure to insecticides during larval growth and development are entirely different stressors and so, their mechanism(s) may differ.

Studies investigating effects of larval nutrition attempted to identify plausible mechanisms by pointing out relationships between adult traits (body size, midgut basement membrane) and competence. Grimstad and Walker (1991) suggested that the strength of the midgut escape barrier in *Ae. triseriatus* was related to size of adult females. These authors hypothesized that the weakening of the midgut escape barrier was caused by nutrient-induced reductions in the basal lamina of the midgut epithelium. These results were consistent with some but not all other investigations of larval nutrient effects on adult vector competence for arboviruses (Table 1). Studies that have identified positive relationships between size and vector competence have suggested that large mosquitoes ingest higher numbers of viruses in blood meals than small mosquitoes (Nasci and Mitchell 1994, Westbrook *et al.* 2010). The expectation in these instances is that higher viral doses associated with larger blood meals increases the probability of midgut infection.

It is likely that size alone is not causally related to altered vector competence, at least when not taking into account the particular larval conditions that produced different sized mosquitoes (e.g. temperature, nutrients, insecticide). The most convincing evidence to support this conclusion comes from studies that produce a range of mosquito sizes with enhanced vector competence. *Ae. aegypti* larvae exposed to insecticide malathion and competition/nutrient limitation resulted in the production of large and small sized adults, respectively, with enhanced viral infection and dissemination relative to control treatments (Muturi *et al.* 2011, unpublished results). These studies demonstrated that in a majority of cases larval environment does indeed alter adult competence for arboviruses, usually with competence enhanced by stress, but the mechanism(s) are probably not simply related directly to mosquito size. Different larval conditions may alter adult vector competence by different mechanisms and these effects may be specific to the virus and mosquito species. Also, for any given larval environment, there may be multiple mechanisms influencing adult competence during the viral infection process, from initial infection in the midgut to transmission. Further advances in identifying plausible mechanisms responsible for altered adult competence have emerged from studies relating differential expression of immune and stress-specific genes to vector competence, suggesting multiple and complex immune responses which need further clarification to identify the details of the immune pathways (Muturi unpublished results).

Conclusions and future directions

This review demonstrates that the larval environment alters adult competence for viruses under laboratory conditions as well as in collections of mosquitoes from the field (Paulson and Hawley 1991). The latter area of research is understudied and we need to address whether larval environment factors in nature alter adult competence. For instance, much of the environmental variation in the field cannot adequately be represented under laboratory conditions. Additionally,

laboratory colonies of mosquitoes, even after only a few generations breeding in a laboratory setting, may not accurately reflect field populations. Laboratory colonization may therefore have consequences for environmental effects on vector competence for arboviruses (e.g. environment x genotype interaction).

Although some studies have attempted to associate adult traits (body size, midgut basement membrane) to competence there appears to be inconsistencies in these relationships. What is the mechanism(s) responsible for the observed changes in adult competence? It is likely that several factors are responsible, especially since it appears that multiple barriers to virus infection and transmission may be influenced by different larval environments (Table 2). Recent advances in molecular biology and our understanding of the expression of immunity related genes have improved the capacity to identify plausible mechanisms responsible for altered adult competence. A further understanding of larval environmental influences on adult competence for viruses will come from the application of these newly developed methods. Molecular studies are valuable as correlates to alterations in vector competence but evidence for causation of actual mechanism(s) probably will require additional experimental manipulations (e.g. gene silencing).

Currently, we have only a rudimentary knowledge of the relationships between larval conditions, altered expression of immunity related genes and viral competence (Muturi unpublished results). Ideally, studies should identify the differential expression of immunity related genes but also the immune pathways including the sequence of immunity events in the mosquito that relate to the viral infection process from initial midgut infection to transmission. These studies will also elucidate whether antiviral molecular responses to infection are similar between different mosquito species and viruses, the latter which differ in genomic structure and replication. Lastly, vector competence is only one parameter that goes into determining disease transmission and studies will need to incorporate other factors as well such as adult life span, biting behaviour, and adult density. Models that incorporate these factors will assist in identifying the net effect of larval environment on disease transmission.

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5. Relevant temperatures in mosquito and malaria biology

Krijn P. Paaijmans and Matthew B. Thomas

Abstract

Most biological process-based models that approximate malaria risk use mean (usually monthly) outdoor air temperature to estimate the various mosquito and parasite life history variables that influence disease transmission intensity. However, mosquitoes, and parasites within them, do not experience 'average temperatures', but are exposed to temperatures that can fluctuate considerably throughout the day. In addition, endophilic mosquitoes will be exposed to indoor temperatures and not directly to outdoor air temperature. Further, mosquito larvae live in aquatic habitats and so again, are not exposed directly to outdoor air temperatures. In this chapter we highlight how these different temperatures can all change malaria risk predictions. To understand malaria dynamics, inform operational control objectives and predict consequences of climate change, we need a better mechanistic understanding of vector-parasite interactions, with improved integration of the biological and environmental parameters at a scale relevant to conditions actually experienced by both mosquitoes and malaria parasites.

Keywords: *Anopheles* vectors, *Plasmodium* malaria, aquatic habitats, daily temperature variability, ectotherms, climate change

Introduction

A proper understanding of the basic biology and ecology of both mosquito vectors and malaria parasites is a prerequisite for development of effective and sustainable malaria control (e.g. Chaves and Koenraadt 2010, Ferguson *et al.* 2010, Marsh 2010). Given the global impact of malaria, it is rather surprising that after more than 100 years of malaria research the mechanistic link between environmental variables, such as temperature, and the risk of malaria remains poorly defined (Lafferty 2009, Paaijmans *et al.* 2009b, 2010b, Pascual *et al.* 2009).

The influence of temperature on malaria can be explored by the basic reproductive number (R_0), which defines the number of cases of a disease that arise from one case of the disease introduced into a population of susceptible hosts. R_0 is commonly described by the formula (Rogers and Randolph 2006):

$$R_0 = \frac{1}{r}\left[\frac{ma^2bcp^{EIP}}{-\ln p}\right] \quad (1)$$

where m is the vector:human ratio, a vector biting frequency, bc transmission coefficients defining vector competence, p daily vector survival rate, EIP the extrinsic incubation or development period of the parasite within the vector, and r the recovery rate of the vertebrate hosts from infection.

Given that 6 out of 7 of these parameters relate in some way to mosquito abundance, ecology or physiology and that mosquitoes are small ectotherm insects, it is clear that the transmission intensity of malaria will be strongly influenced by environmental temperature (see e.g. Craig *et al.* 1999, Guerra *et al.* 2008, Harvell *et al.* 2002, Parham and Michael 2010, Patz and Olson 2006, Rogers and Randolph 2006).

W. Takken and C.J.M. Koenraadt (eds.), *Ecology of parasite-vector interactions*, Ecology and control of vector-borne diseases 3, DOI 10.3920/978-90-8686-744-8_5, © Wageningen Academic Publishers 2013

In general, ectothermic performance across temperature is traditionally summarized as a nonlinear asymmetric curve (Brière *et al.* 1999, Lactin *et al.* 1995, Logan *et al.* 1976). This non-linear relationship has been described for various malaria mosquito and parasite life-history characteristics, such as immature mosquito development (Bayoh and Lindsay 2003), length of the gonotrophic cycle (Lardeux *et al.* 2008) and parasite development time (Ikemoto 2008, Paaijmans *et al.* 2009b) (Figure 1).

Most biological process-based models that approximate malaria risk use mean (usually monthly) outdoor air temperature to estimate various variables that influence disease transmission intensity (Craig *et al.* 1999, Guerra *et al.* 2008, Guerra *et al.* 2010, Killeen *et al.* 2000, Martens *et al.* 1999, Rogers and Randolph 2000). The aim of this chapter is to critically revisit this approach by exploring whether outdoor mean air temperature is the appropriate environmental driver for understanding different aspects of mosquito and parasite biology. Specifically, for traits relating to the adult mosquitoes we consider the influence of mean vs. variable temperatures and outdoor vs. indoor temperatures. Additionally, for the juvenile mosquitoes we consider the influence of air vs. water temperatures.

Overview of methods

Temperature-dependent physiological models

We use two types of temperature-dependent models (i.e. the widely-applied linear rate models and nonlinear asymmetric thermodynamic models (Figure 1)) to compare the effects of these different environmental metrics on various mosquito/malaria life history traits that influence malaria transmission, considering both current and future climate scenarios (note we consider climatic change to include changes in local conditions due to factors such as deforestation (Afrane *et al.* 2008, Lindblade *et al.* 2000) or changes in building structure (Atieli *et al.* 2009, Okech *et al.* 2004a,b)).

To examine the effects of temperature on mosquito/parasite life history traits we used a range of published thermal performance curves (Figure 1). For malaria parasite development we compared

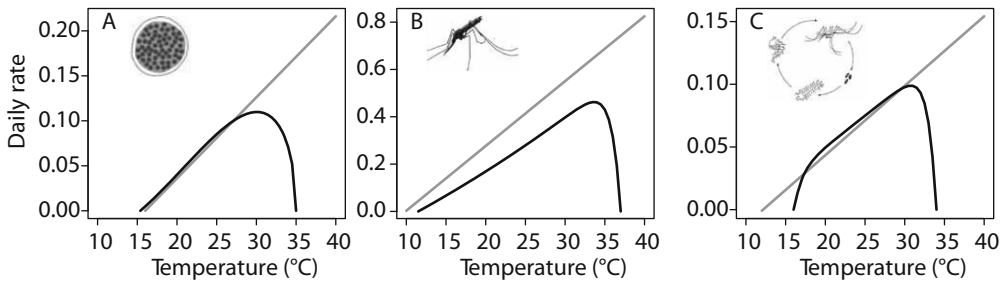

Figure 1. Published thermal performance curves showing the relationship between temperature (in °C) and (A) parasite development rate, (B) the inverse of the gonotrophic cycle length, (C) larval development rate. Grey lines show linear thermal performance curves presented by (A, B) Detinova (1962) and (C) Craig et al. *(1999). Black lines represent the non-linear thermal performance curves presented by (A) Paaijmans* et al. *(2009b), (B) Lardeux* et al. *(2008) and (C) Bayoh and Lindsay (2003).*

the linear rate model of Detinova (1962) with the equivalent non-linear thermodynamic model of Paaijmans *et al.* (2009b). For the gonotrophic cycle (or feeding frequency) we used the linear rate model of Detinova (1962) and the non-linear thermodynamic model of Lardeux *et al.* (2008). Finally, for larval development we compared the linear rate model of Craig *et al.* (1999) with the non-linear thermodynamic model of Bayoh and Lindsay (2003).

Rates were calculated at 30 min intervals using either the mean temperature, or the actual temperature at that given time-point (see below). Growth rates were accumulated until they reached a value of 1, which defines the completion of the rate process (see Paaijmans *et al.* 2009b).

Environmental temperature data

The minimum and maximum air temperature data used in the Section 'Mean vs. variable temperature' were obtained from the National Climatic Data Center (http://www.ncdc.noaa.gov/oa/mpp/freedata.html). Mean monthly air temperature and mean monthly daily temperature range (DTR) were calculated at five met-stations (Kericho, Nakuru, Kisumu, Voi and Khartoum) in East Africa for March 2001. The indoor and outdoor minimum and maximum temperature data that are used in the Section 'Outdoor vs. indoor temperature' were recorded in Tanzania by Bødker *et al.* (2003). Air temperatures and water temperatures reported in the Section 'Water vs. air temperature' were derived from the studies of Paaijmans *et al.* (2008, 2010a).

Modeling daily temperature variation between minimum and maximum temperatures

The phase and form of the diurnal rhythm of the air temperature (T) is given by a sinusoidal progression during daytime (Equation 2) and a decreasing exponential curve during the night (Equation 3) (Parton and Logan 1981):

$$T = T_{min} + (T_{max} - T_{min}) \sin\left[\pi \frac{t - 12 + D/2}{D + 2p}\right] \tag{2}$$

for $t_{rise} \leq t < t_{set}$

$$T = \frac{T_{min} - T_{set} \exp(-N/\tau) + (T_{set} - T_{min}) \exp(-(t - t_{set})/\tau)}{1 - \exp(-N/\tau)} \tag{3}$$

for $t_{set} \leq t < t_{rise}$

where T_{min} and T_{max} (°C) are the minimum and maximum daily air temperature, respectively, t (hours) the time, D (12 hours) the daylength, p (hours) the time duration between solar noon and maximum air temperature, t_{rise} (hours) the time of sunrise, t_{set} (hours) the time of sunset, T_{set} (°C) the temperature at sunset, N (hours) the duration of the night and τ the nocturnal time constant.

Using the reported minimum and maximum temperatures in the Sections 'Mean vs. variable' and 'outdoor vs. indoor' temperature, we modeled the air temperature at 30 min intervals. Mean temperatures are calculated with these 30 min interval data, and not by taking the mean of the minimum and maximum temperature.

Effects of temperature on transmission intensity (basic reproduction rate) of malaria

Temperature affects multiple parameters that comprise R_0. However, for simplicity, in the following sections we consider changes in single parameters only. In Sections 'Mean vs. variable' and ' outdoor vs. indoor' temperature we consider the extrinsic incubation period and feeding frequency (the latter is given by the inverse of the gonotrophic cycle length). Unless stated, we follow others and assume a median daily mosquito survivorship of 0.860 (Kiszewski *et al.* 2004) and a maximum mosquito lifespan of 31 days (Guerra *et al.* 2008).

In the Section 'Water vs. air temperature' we consider changes in larval development period and its effect on population growth rate (which will affect the vector:host ratio). For this we calculate the intrinsic rate of increase, *r*, using the analytical approximation:

$$r = \ln R_n / G \quad (4)$$

where R_n the net reproductive rate and *G* is mean length of a generation (Gotelli 2001). Parameter estimates for *G* and R_n were derived from the study of Afrane *et al.* (2006) who measured adult longevity and daily reproductive fitness for *Anopheles gambiae* Giles at a lowland and highland site in Western Kenya during the rainy season when mean air temperatures were almost identical to those of the current study. Net reproductive rate (R_n) was estimated directly by Afrane *et al.* (2006) with values of R_n=346.0 and R_n=434.8 for the lowland and highland sites, respectively. Mean length of a generation was calculated as the median time for adult reproduction (24.7 and 20.7 days for the lowland and highland sites, respectively, data derived from Afrane *et al* (2006)) plus the relevant duration of larval development from this study to obtain the mean length of a complete generation (i.e. including all immature stages).

The reported changes in R_0 or *r* might be conservative if other mosquito and parasite life-history traits scale similarly with temperature. However, there is a possibility that there are trade-offs between traits, whereby a gain in one trait could be offset be losses in another. For example, in many biological systems, faster development at higher temperatures is costly and can trade-off with survival. For several parasite species it has been shown that development rates interact with survival (e.g. Kutz *et al.* 2009, Studer *et al.* 2010), and for *An. gambiae*, faster immature development at warmer temperatures is offset by fewer numbers surviving (Bayoh and Lindsay 2003). However, potential trade-offs between traits remain largely unexplored for malaria so they are not further considered here. Nonetheless, understanding the potentially complex effects of temperature across multiple interacting traits is an important area for further research.

Mean vs. variable temperature

Mosquitoes and parasites are poikilothermic, and therefore their temperature will track that of their direct surrounding environment. The daily temperature range (DTR; the difference between the daily minimum and maximum temperature) can easily be larger than 10 °C in Africa (Geerts 2003, Paaijmans *et al.* 2010b) (Table 1). Thus, mosquitoes, and parasites within them, do not experience 'average temperatures', but are exposed to temperatures that can fluctuate considerably throughout the day.

In an earlier theoretical study we assessed the potential effects of temperature fluctuation on development of the malaria parasites within the mosquito and revealed that parasite development is expected to be faster under fluctuating low temperatures, and slower under fluctuating high

Table 1. Mean monthly air temperature and mean monthly daily temperature range (DTR) as recorded in March 2001 at five meteorological stations in East Africa. Data obtained from the National Climatic Data Center.

	Kericho	Nakuru	Kisumu	Voi	Khartoum
altitude	1,976 m	1,901 m	1,146 m	579 m	380 m
N[1]	23 days	30 days	31 days	28 days	29 days
Mean T_a	16.7 °C	19.9 °C	23.9 °C	27.7 °C	29.5 °C
DTR	11.6 °C	14.9 °C	13.0 °C	13.3 °C	15.9 °C

[1] Number of days with available temperature (minimum and maximum) data in March 2001.

temperatures, compared with the respective baseline mean temperatures (Paaijmans *et al.* 2009b). In follow-up experimental studies we showed that this theory is robust, and extends to various aspects of life-history of the mosquito (Paaijmans *et al.* 2010b).

The observed non-linear rate summation effect is characterized as the Kaufmann effect (Kaufmann 1932) or Jensen's inequality (see e.g. Ruel and Ayres 1999) and has long been recognized in a range of host-pathogen/parasite systems (see e.g. Arthurs *et al.* 2003, Giannakou *et al.* 1999, Ruissen *et al.* 1993, Scherm and Van Bruggen 1994, Xu 1996) and for many insect-related rate processes in general (Worner 1992), but until recently has not been considered with respect to mosquitoes and pathogens.

To show the potential effects of daily temperature variability on parasite development and feeding frequency, we selected temperature data sets from five locations in East Africa (Table 1, Figure 2A) The extent of malaria transmission in these areas is not uniquely determined by temperature but we select them here to illustrate differential effects of temperature across diverse environments.

Extrinsic incubation period

As indicated previously, daily temperature fluctuation around cool temperatures acts to speed up parasite development relative to the baseline mean temperatures. This can be seen in the cooler areas of Kericho and Nakuru, with effects getting more pronounced as temperatures decline (nonlinear model; Figure 2B).

In Nakuru, parasites are predicted to fully develop in 26.6 days (i.e. within the maximum mosquito lifespan) when temperature fluctuates, but to take 32.5 days (i.e. beyond the maximum mosquito lifespan) at the baseline mean temperature (Figure 2B).

If we relax the mosquito lifespan threshold, and apply a maximum mosquito lifespan of 56 days as used by Craig *et al.* (1999), parasite development will be completed within mosquito lifespan in Kericho under variable temperature conditions (51.9 days), but not at the baseline mean temperature (189.2 days). This means that at the fringes of malaria transmission, fluctuation makes transmission possible at lower mean temperature.

Fluctuation around warmer temperatures has the reverse effect, slowing down growth rate relative to the equivalent constant mean temperatures. This effect is more pronounced as mean

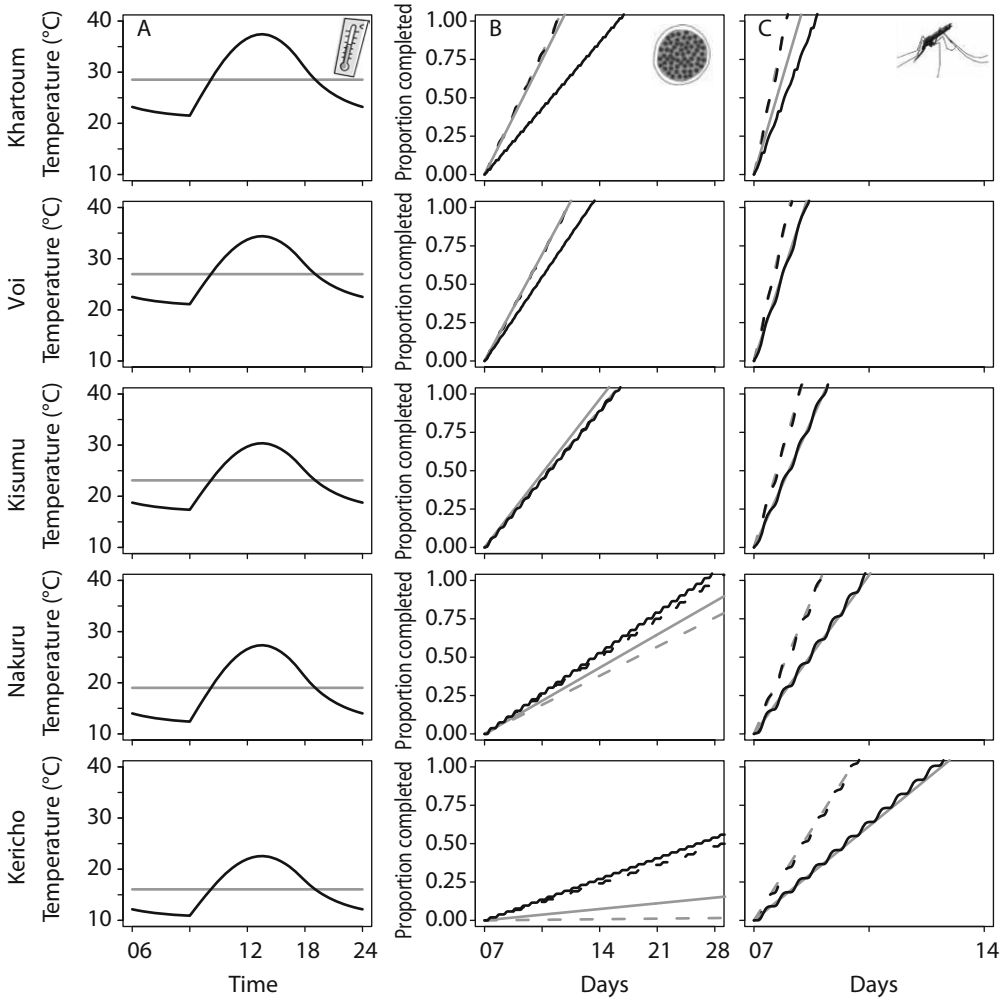

Figure 2. (A) Mean monthly temperatures (°C; grey lines) and modeled mean monthly daily temperature fluctuations (°C; black lines) at five different met-stations in East-Africa (March 2001). (B) Parasite development times, and (C) gonotrophic cycle lengths, at these locations, as estimated by the mean temperature (grey lines) or by taking daily temperature fluctuation into account (black lines). Dashed lines show development as predicted with the linear models, solid lines as predicted with the non-linear models. Development/cycle is completed when the proportion equals 1. Drawings of oocysts were generated by the US Centers for Disease Control and Prevention (http://www.dpd.cdc.gov/dpdx/HTML/Malaria.htm).

temperatures increase (Figure 2B, Kisumu: lengthened by 1.2 days, Voi by 2.7 days, and Khartoum by 6.9 days).

Qualitatively similar results are observed with the linear rate development model of Detinova (Figure 2B) when temperature fluctuates around lower mean temperatures (for Kericho and Nakuru). However, there is no difference in EIP estimates when applying the mean temperature or daily temperature variability in the warmer areas. This is due to the fact that temperature fluctuates over the linear part of the growth curve, so gains during daytime are offset by losses during nighttime. Furthermore, there is no upper temperature threshold in this type of model, so growth rate continues to increase with temperature.

Gonotrophic cycle length

The effect of daily temperature variation on the length of the gonotrophic cycle is much less pronounced than its effects on *EIP*. In Kericho fluctuation reduces the gonotrophic cycle by 0.5 days, whereas in Khartoum fluctuation creates a delay of 0.9 days, compared with the baseline mean temperatures (nonlinear model; Figure 2C).

These smaller effects are caused by the shape of the temperature-dependent model, which spans a wider temperature range than that of the *EIP* (Figure 1A,B). So in most of the selected areas, temperature will fluctuate over the linear part of the thermal performance curve.

Detinova's equation predicts no difference in gonotrophic cycle length when temperature variability is considered, due to a combination of a low minimum threshold temperature (9.9 °C) and temperatures fluctuating along the linear part of the growth curve (Figure 2C). However, the accuracy of the Detinova model is questionable since it predicts very short gonotrophic cycles even at cooler temperatures (e.g. 6 and 4 days in Kericho and Nakuru, respectively), which is at odds with empirical data (Afrane *et al.* 2005, Lardeux *et al.* 2008).

Changing climate

The relative effects of increases in mean temperature are likely to be less than expected when daily temperature fluctuation is taken into account (see Paaijmans *et al.* 2009b). We have illustrated this further in Figure 3, where we consider influence of temperature variation on a generic non-linear thermal performance curve (Figure 3A) (this curve is not specific for any particular life history trait but is used for illustration).

When we consider a longer-term prospective scenario with increases in mean monthly temperature of 3.2 °C, corresponding to the median increase in terrestrial temperature predicted by the IPCC for the months March-May in East-Africa by 2100 (Christensen *et al.* 2007), we see that at cooler temperatures the relative change in rate is much less at larger DTRs compared to the change in rate at the baseline constant temperature (black arrows in Figure 3B). However, it is important to note that the rate itself is already higher.

At higher temperatures, it is not necessarily the case that climate warming will only result in higher rates: no effect of further warming could be observed at certain DTRs (e.g. upper grey arrow in Figure 3B), and at some mean temperature-DTR combinations a decrease in rate could be observed under climate warming.

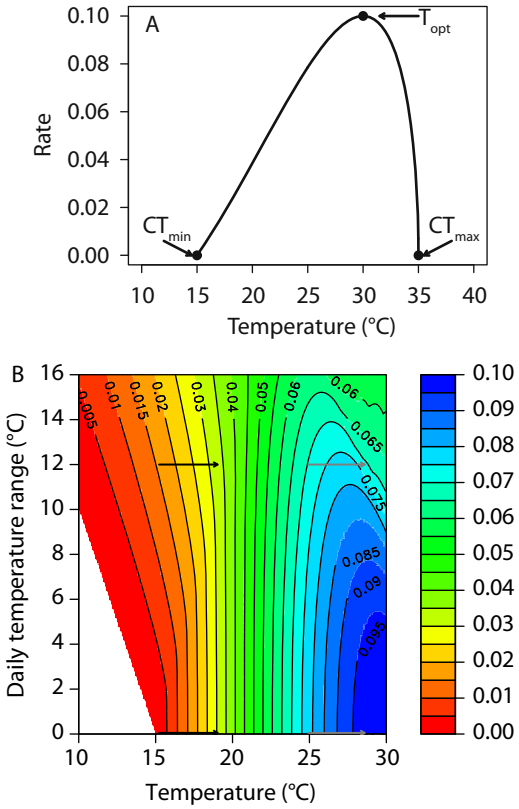

Figure 3. (A) Typical asymmetrical thermal ectotherm performance curve, indicating lower (CT_{min}) and upper (CT_{max}) critical temperatures for a given rate process and the temperature at which performance is maximum (T_{opt}). For illustration purposes we have selected a window of operation of 20 °C, which is typical for many insect species (Dixon et al. *2009), and a rate process that completes in 10 days at T_{opt}. (B) The rate of the same exemplary life-history trait across a range of mean temperatures (10-30 °C) and a wide range of daily temperature ranges (0-16 °C). Arrows indicate a shift in the mean temperature of approx. 3.2 °C due to global warming (see text).*

An additional complication is that global warming is unlikely to result in a symmetrical shift in minimum and maximum temperatures. Several studies predict proportionately greater increases in daily minima than in daily maxima, resulting in decreases in DTR (Easterling *et al.* 1997, King'uyu *et al.* 2000, Lobell *et al.* 2007), although the reverse is possible at local scales (Hulme *et al.* 2001, King'uyu *et al.* 2000). Defining the nature of these changes at particular sites will be important for predicting local changes in disease burdens because changes in DTR can exacerbate or mitigate the influence of increases in mean temperatures, depending on initial starting conditions (Paaijmans *et al.* 2009b).

Short-term changes in climate, due to e.g. land-use changes, could also result in an asymmetrical shift of the daily minimum and maximum temperature. Work by Afrane *et al.* (2005) showed that both the mean and maximum temperatures increased substantially following deforestation in a

highland area in western Kenya. Again, diurnal temperature fluctuation could reduce the relative impact of such changes in mean temperature on life-history characteristics, although the non-linearities, together with changes in DTR, make patterns complex.

In summary, neither the essential transmission parameters nor the upper or lower temperature thresholds for transmission can be estimated with the mean temperature alone. Models that ignore diurnal variation overestimate malaria risk in warmer environments and underestimate risk in cooler environments, and will tend to exaggerate the impact of climate change.

Outdoor vs. indoor temperature

The gonotrophic cycle of an anopheline mosquito can be as short as two days, but could take over a week, depending on temperature (Afrane *et al.* 2005, Lardeux *et al.* 2008, Rúa *et al.* 2005). As oviposition, host-seeking and blood-meal uptake are likely to happen in a single night, mosquitoes can spend a considerable part of the gonotrophic cycle (and hence their adult life) resting, during which the blood meal is digested and eggs develop.

An. gambiae s.s., arguably the most important malaria vector in sub-Saharan Africa, will spend a considerable time indoors, as it typically rests indoors (endophily) (Githeko *et al.* 1996b, Faye *et al.* 1997, Highton *et al.* 1979, Mnzava *et al.* 1995, Service 1970), although the reverse (exophily) has been reported (Bockarie *et al.* 1994; Mahande *et al.* 2007).

Unfortunately, there are only a few studies that actually measure the mean indoor and outdoor temperature simultaneously, and even fewer studies that keep track of the actual daily temperature variability in both environments. A generality in those studies is that the mean indoor temperature in traditional houses tends to be a few degrees Celsius higher than the outdoor temperature (Afrane *et al.* 2005, 2006, 2007, 2008, Alonso *et al.* 2011, Bødker *et al.* 2003, Garnham 1945, 1948, Minakawa *et al.* 2006), and this observation is qualitatively robust to changes in altitude. Thus, endophilic mosquitoes, and parasites within them, will be exposed to warmer temperatures than the outdoor air temperature.

Using indoor and outdoor temperature data from a study carried out in Tanzania (Bødker *et al.* 2003) (Figure 4A), we assessed the effects of these different microclimate datasets on the extrinsic incubation period of the parasite and gonotrophic cycle length of the mosquito.

Extrinsic incubation period

When we compare mean outdoor with mean indoor temperatures, applying a nonlinear model, we see that warmer indoor temperatures result in faster parasite development, with the difference getting larger with altitude (3.6 and 12.2 days faster at 640 and 1,040 m, respectively). At 1,686 m *EIP* is successfully completed indoors (30.1 days), whereas outdoor temperatures are too low for successful development (Figure 4B).

Comparing estimates based on the outdoor and indoor actual temperature fluctuations yields qualitatively similar results (Figure 4B). Also, incorporating DTR into the model has no real effect on *EIP* when compared to the baseline mean temperatures, but the reasons for the outdoor and indoor environment are different. Outdoor temperatures fluctuate along the linear part of the growth model. Indoors the DTRs are simply too small to have any impact.

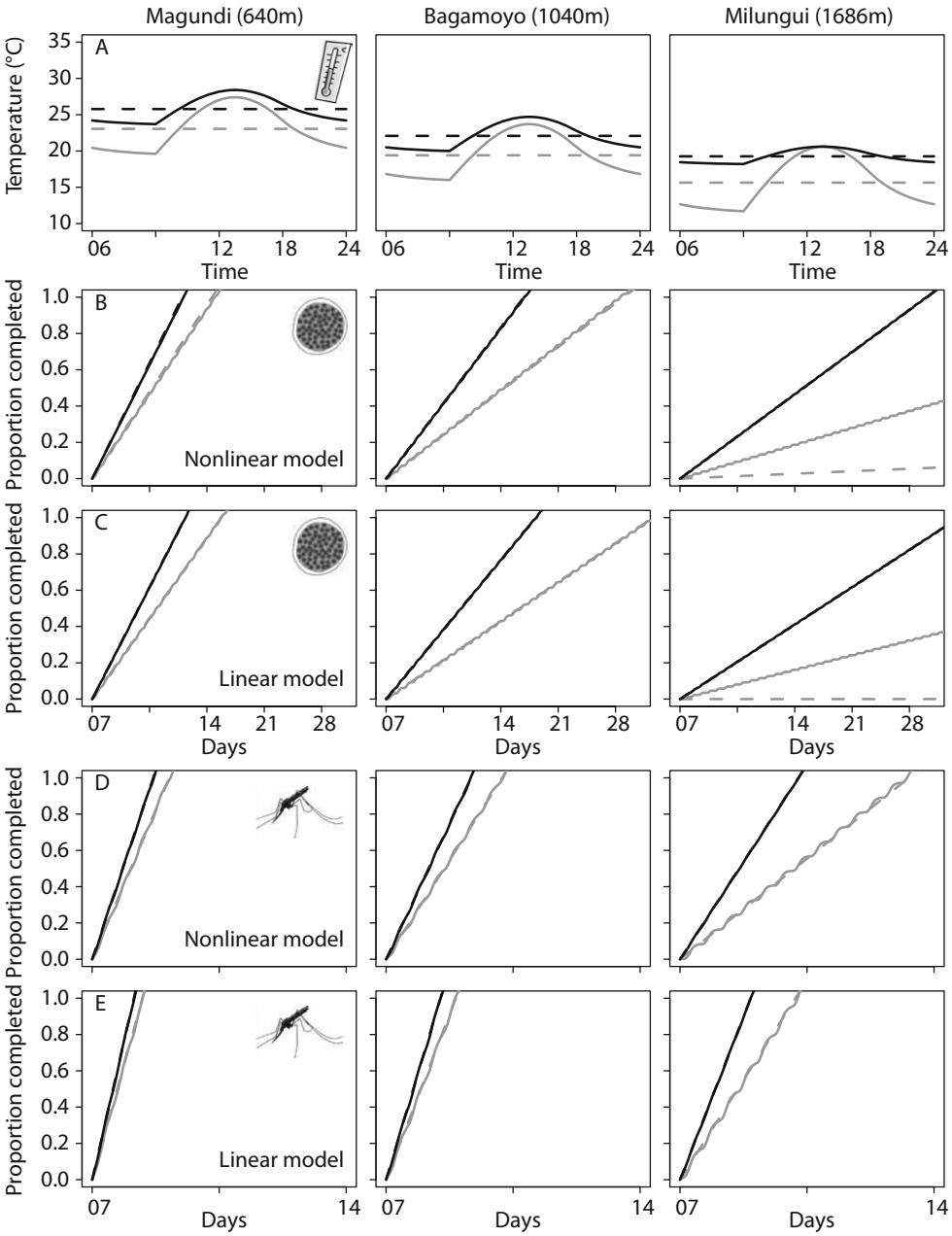

Figure 4. (A) Mean temperatures (°C; dashed lines) and modeled daily temperature dynamics (°C; solid lines) outside (°C; grey lines) and inside (°C; black lines) human dwellings, as recorded by Bødker et al. *(2003). (B, C) Parasite development times, and (D, E) gonotrophic cycle length, are modeled using the mean outdoor temperature (dashed grey lines), mean indoor temperature (dotted black lines) or the daily temperature fluctuation recorded outdoors (solid grey lines) or indoors (solid black lines). (B, D) Estimates with the non-linear models, (C, E) Estimates with the linear models. Development/cycle is completed when the proportion equals 1. Drawings of oocysts are generated by the US Centers for Disease Control and Prevention (http://www.dpd.cdc.gov/dpdx/HTML/Malaria.htm).*

Having said that, large DTRs of 10-15 °C are observed indoors, including in highland environments (Okech *et al.* 2004a; Okech *et al.* 2004b; Afrane *et al.* 2006; Afrane *et al.* 2008). In the study by Bødker *et al.* (2003) that we used as an example here, temperature data are averaged over a period of several months, masking any larger day-to-day variations in DTR.

The linear Detinova model predicts that parasites will not develop successfully at 1,686 m (for neither indoor nor outdoor temperature data) (Figure 4C). At 1,040 m parasites will similarly not be able to complete development in exophilic mosquitoes, whereas the non-linear model predicts that malaria will fully develop in exo- and endophilic mosquitoes and in endophilic mosquitoes at 1,040 and 1,686 m, respectively. The difference between the two models is due to a small difference in the lower minimum threshold temperature for parasite development (Figure 1A).

Gonotrophic cycle length

Again, as seen in the Section 'Mean vs. variable temperature', a qualitatively similar outcome is observed for the gonotrophic cycle length. Compared to indoor conditions, and applying a nonlinear model, outdoor temperatures lengthen the gonotrophic cycle by 0.9 and 1.7 days at 640, 1,040 m altitude, respectively, and predictions based on the mean temperature are similar to those based on the daily temperature variability (Figure 4D). At 1,686 m, the gonotrophic cycle takes 6.1 (mean) or 5.9 days (DTR) longer to complete, compared to the indoor temperature. The reasons for the small differences in the length of the gonotrophic cycle between mean vs. fluctuating temperature are similar to those discussed in the previous Section 'Mean vs. variable temperature'. The Detinova curve again predicts much shorter gonotrophic cycles (see Section 'Mean vs. variable temperature', Figure 4E).

Changing climate

The potential effects of a changing climate on the outdoor air temperature, and hence on mosquito and parasite life-history characteristics, is described in the previous section. How indoor microclimate will be affected by longer-term climate change is hard to predict. There are multiple factors that will ultimately play a role. Humans can manipulate their direct thermal environment by changing the nature of the building structure (Atieli *et al.* 2009, Okech *et al.* 2004a,b), or its surroundings (Afrane *et al.* 2005, 2006, 2007, 2008). Mosquitoes might also react to unfavourable indoor conditions; in a study exploring the effects of steadily increasing temperature on behaviour (Kirby and Lindsay 2004), *An. gambiae* and *An. arabiensis* Patton exhibited escape responses at 33 °C and 35.7 °C, respectively.

Moreover, the use of insecticides on bednets (ITNs) (Lindsay *et al.* 1991, Lines *et al.* 1987, Mbogo *et al.* 1996, Miller *et al.* 1991), on eaves curtains (Githeko *et al.* 1996a) or by spraying insecticides indoors (IRS) (Service *et al.* 1978), all have the potential to keep/drive malaria vectors outdoors. This change in behaviour means that vectors will immediately be exposed to different microclimatic conditions.

The well-documented consequences of deforestation on climate provide us with an insight of climate warming on a very short temporal scale. Deforestation in cooler highland areas in western Kenya result in warmer mean and maximum indoor temperatures, increasing the indoor DTR (Afrane *et al.* 2005, 2006). Increases in the minimum temperature have also been observed after deforestation (Afrane *et al.* 2008). Such changes in temperature could result in increased parasite development rates and shorter gonotrophic cycles. Both have been observed empirically: for *EIP*, see Afrane *et al.* (2008) and for gonotrophic cycle length, see Afrane *et al.* (2005).

In summary, existing models that use outdoor air temperatures will tend to underestimate the speed of rate processes such as parasite development, blood meal digestion and egg-production of indoor resting mosquito populations, with differences being larger in cooler environments. The impact of climate warming is hard to assess due to many uncertainties. Clearly we need a better understanding of mosquito whereabouts and the associated microclimate.

Water vs. air temperature

The relationship between immature mosquito biology and temperature is central to numerous studies exploring the temporal and/or spatial patterns of malaria risk (Bayoh and Lindsay 2003, Craig *et al.* 1999, Ebi *et al.* 2005, Hoshen and Morse 2004, Ikemoto 2008, Pascual *et al.* 2006). A feature of nearly all such studies is the use of mean ambient air temperature to drive the relevant growth processes. While this might be appropriate for processes relating to transmission by the adult mosquito (but see Sections 'Mean vs. variable' and 'outdoor vs. indoor' temperature), the immature stages of malaria mosquitoes, such as *An. gambiae*, inhabit aquatic environments such as small, transient, sunlit pools (e.g. Gimnig *et al.* 2001, Mutuku *et al.* 2006).

Water temperatures are generally higher than corresponding air temperatures throughout most of the day, with mean water temperature exceeding mean air temperature by a few degrees Celsius, in both lowland and highland areas (Table 2, Figure 5A, Minakawa *et al.* 2006, Munga *et al.* 2005, Munga *et al.* 2006, Paaijmans *et al.* 2008, 2010a). Given the fundamental fact that mosquito larvae live in aquatic and not terrestrial habitats, immature mosquitoes do not experience air temperatures, but are exposed to warmer water temperatures.

To illustrate the difference in impact of water vs. air temperature on mosquito biology, we estimated immature development using both linear and non-linear development models (Figure 1C), driven by temperatures recorded in a lowland (Kisian) and highland area (Fort Ternan) in western Kenya. A day with predominant clear sky was selected. Mean air and water temperature, as well as daily temperature ranges are given in Table 2 and Figure 5A.

Table 2. Mean air and water temperatures, and their daily temperature ranges, as recorded on a day with no overcast in a lowland and a highland site in western Kenya.

Kisian (1,126 m)	29 March 2005
Total incoming short wave radiation	26.8 MJ/m^2
Mean air temperature	23.8 °C
DTRair	13.3 °C
Mean water temperature	28.4 °C
DTRwater	11.9 °C
Fort Ternan (1,552 m)	22 May 2006
Total incoming short wave radiation	26.7 MJ/m^2
Mean temperature	19.5 °C
DTR	12.7 °C
Mean water temperature	25.1 °C
DTRwater	12.1 °C

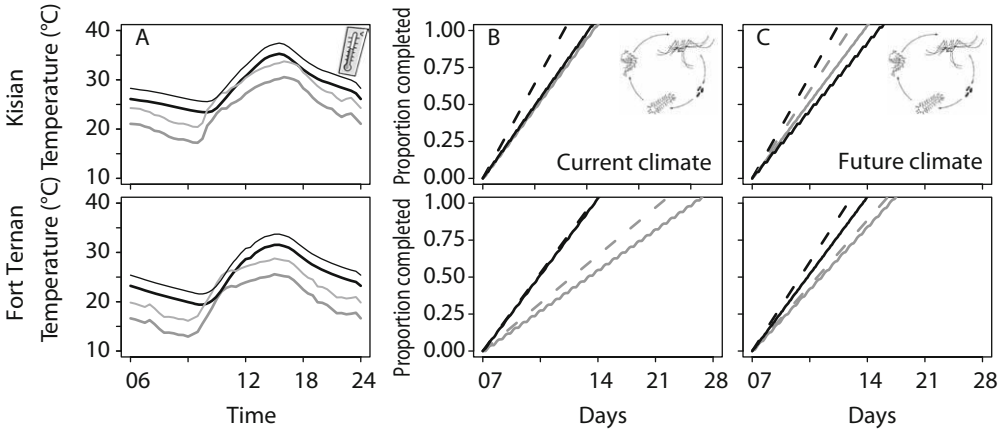

Figure 5. (A) Air temperature (grey lines) and water temperature dynamics (black lines) in a typical mosquito breeding puddle as recorded in Kisian and Fort Ternan, a lowland and a highland site, respectively, in western Kenya. Thick lines are actual measured temperatures, thin lines the forecasted temperatures (see text). (B, C) Immature development times predicted with the non-linear model using the mean temperature (dashed lines) or the daily temperature variation (solid lines) of the air (grey lines) or water temperatures (black lines). (C) Similar to B, but now showing the effects of climate warming on larval development times. Development is completed when the proportion equals 1.

Water temperatures in Kisian and Fort Ternan were higher than corresponding air temperatures throughout the day, with mean water temperature exceeding mean air temperature by 4.6 and 5.6 °C, respectively.

Larval development times

Larval development rates as estimated with mean water temperatures are much higher than estimates that are based on mean air temperatures. Higher mean water temperatures shorten larval development times by 3.6 days in the lowland and 8.3 days in the highland area (nonlinear model; Figure 5B).

When daily water temperature variability is included in the model, we get qualitatively similar results: larval duration is shortened by 0.6 days in Kisian compared to fluctuating air temperatures. The fact that immature mosquito develop slower under variable conditions, compared to constant conditions, is caused by a period of high water temperatures during daytime, which slow down and even inhibit development for a short period during the day (Figure 5B). In the highland area larval duration is shortened by 12 days, compared with estimates based on the variable air temperature (Figure 5B). This is not only the result of the air temperature being lower than the water temperature, and hence predicting lower development rates; another factor is that air temperatures drop below the minimum critical temperature for larval development (CTmin) during night-time, resulting in a shorter period of growth compared to estimates that are based on fluctuating water temperatures.

The immature development times that are derived using aquatic temperatures in the current study are consistent with the observed larval development times in several field studies in western Kenya (Gimnig *et al.* 2002, Minakawa *et al.* 2006, Munga *et al.* 2006, Paaijmans *et al.* 2009a).

The linear model provides a qualitatively similar outcome to the nonlinear model described above (4.4 days reduction in development time in the lowlands, and 10.8 days in the highlands, under both mean and variable conditions; data not shown), as the models overlap over a large part of the operable temperature range. The linear model does, however, not capture the slower development (as described above) at the lower temperature end due to differences in model-shape at cooler temperatures (Figure 1C). Due to a combination of (1) there being no upper temperature threshold in the linear model, and (2) the fact that temperatures observed in our field sites fluctuate over the linear part of the growth curve, the linear model predicts similar development rates in both constant and variable environments.

Changing climate

The relative impact of temperature warming is likely to be less when the relevant water temperatures are taken into account. This is because (1) water temperature of typical malaria mosquito breeding sites is higher than the surrounding air temperature, and therefore the baseline larval development and population growth rates themselves are much higher, and will therefore see a smaller increase compared to estimates that are based on the air temperature (Paaijmans *et al.* 2010a) and (2) the actual increase in water temperature is expected to be less than the actual increase in air temperature since the slopes of the regression lines describing the relationship between water and air temperature are <1 (Paaijmans *et al.* 2010a).

To illustrate this we consider a longer-term prospective scenario with increases in mean monthly air temperature of 3.2 °C (see Section 'Mean vs. variable temperature'). The actual increase in water temperature is expected to be to be 3.2×0.677 (slope) = 2.2 °C. For simplicity we assumed no changes in the diurnal ranges of air and water temperatures (Figure 5A).

Adding the projected increase of 3.2 °C in temperature to current mean air temperatures dramatically shortens larval development times (Figure 5C) and increase population growth rates. Again, however, the magnitude of these effects is greatly reduced when the relationship between air and water temperature is taken into account; instead of decreases in larval development times of 2.7 days in the lowland and 5.8 days in the highland area, development times are predicted to shorten by only 0.8 days and 1.7 days, respectively.

As a consequence, the increases in population growth rates compared to present day are predicted to be in the order of 2.3% in the lowland area and 5.1% in the highland area rather than 7.4% and 15.9%, respectively, as predicted with the mean air temperature.

When temperature variability is taken into account similar patterns emerge; instead of decreases in larval development times of 1.3 days in the lowland and 8.9 days in the highland area, development times are predicted to lengthen by 1.1 days and shorten by 0.1 days, respectively. The longer larval development time in the lowlands is caused by the fact that larvae are exposed even longer to unfavourable temperatures during daytime, which inhibit development. This results in a decrease in population growth rate of 2.8% compared to present day, rather than an increase of 3.3% in the lowland area. In the highland area there is an increase in the intrinsic rate of increase of only 0.4% rather than the 23.7% that is predicted with the variable air temperature.

Essentially, although warming is expected to increase growth rate via effects on larval development, the relative change is expected to be much less, and could even be reversed, when the relevant water temperatures are considered.

Unfortunately, we have a poor understanding of how climate warming will eventually change the daily temperature dynamics of mosquito breeding habitats. On shorter time scales, changes in microclimate due to deforestation (or swamp reclamation) have been observed to increase mean temperatures in larval habitats (increase of 4.8 °C in the lowland and 4.7 °C in the highland area), with larger increases in the daily maximum water temperature than in the daily minimum water temperature. In these warmer pools, larval development times are shortened by several days in the lowlands, and by a week or more in a cooler highland area (Minakawa *et al.* 2006).

In summary, air temperature alone does not provide an appropriate variable for estimating immature mosquito development or for setting threshold temperatures. Existing models will tend to underestimate mosquito population growth under current conditions. On the other hand, the relative increases in larval development rates predicted due to climate change are substantially less. Again, existing models may overestimate relative increases in population growth under future climate change.

Concluding remarks

This book chapter highlights how the standard use of the mean outdoor air temperature in malaria risk models fails to capture important features of the actual microclimate experienced by mosquitoes. Immature and adult mosquitoes, as well as the parasites within the adults, are likely to be exposed to considerable different temperatures than currently taken into account. In addition, daily temperature variations (in outdoor and indoor air temperature, as well as in water temperature) and the type of model (linear vs. non-linear) that is used clearly affect malaria risk predictions as well.

Given the need to understand malaria dynamics for setting operational control objectives and for predicting consequences of climate change, this chapter highlights an urgent need to develop a better mechanistic understanding of vector-parasite interactions, with improved integration of the biological and environmental parameters at a scale relevant to conditions actually experienced by both mosquitoes and malaria parasites.

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6. Evolutionary aspects of *Anopheles-Plasmodium* interactions

Louis Lambrechts and Jacob C. Koella

Abstract

Interactions between *Plasmodium* parasites and their *Anopheles* vectors are central to the epidemiology of human malaria. This chapter highlights how an evolutionary perspective on *Anopheles-Plasmodium* interactions can provide important insights into the history, distribution and dynamics of malaria transmission. It focuses on three aspects: (1) the macro-evolutionary history of malaria parasites in relation with their vectors, (2) the micro-evolutionary mechanisms shaping mosquito-parasite interactions and their impact on malaria transmission, and (3) the contribution of evolutionary concepts in the assessment of novel strategies to control malaria. The geographical distribution of diverse anopheline species and populations has played an important role in the past and present distribution of malaria. In particular, speciation processes and genetic differentiation of vectors may have been important drivers of the evolution of human malaria parasites. A full understanding of the epidemiology of malaria also requires careful consideration of the micro-evolutionary relationships between mosquitoes and malaria parasites. Malaria parasites have evolved, for example, to manipulate several parameters of the vector biology that are expected to increase their transmission. Finally, an evolutionary approach is useful for assessing the feasibility of innovative malaria control strategies such as the release of transgenic mosquitoes. Considering the epidemiological feedback and the evolutionary response of wild mosquito and parasite populations is crucial for predicting the evolutionary trajectory of such a control measure. A major challenge for future research is to obtain quantitative, epidemiologically relevant estimates of the critical parameters underlying *Anopheles-Plasmodium* interactions in natural systems.

Keywords: vector-parasite interactions, malaria epidemiology, parasite manipulation, evolutionary feedback, genetically modified mosquitoes

Introduction

This chapter deals with evolutionary aspects of the interactions between malaria parasites and their mosquito vectors. Rather than exhaustively covering the topic, it describes examples of three aspects: macro-evolutionary patterns, the role of evolutionary ecology in shaping epidemiology, and the use of evolutionary ideas for control. The section 'Role of mosquitoes in evolutionary history of *Plasmodium*' reviews approaches of population biology and phylogenetics that have shed light on the macro-evolutionary history of malaria parasites in relation with their mosquito vectors. In particular, it describes the association of mammalian *Plasmodium* species with their specialization to *Anopheles* mosquito vectors, and the link between anopheline diversity and the current distribution of *Plasmodium* species and populations. The next Section 'Evolutionary ecology of mosquito-malaria interactions' describes the micro-evolutionary mechanisms shaping interactions between *Plasmodium* and mosquitoes and their impact on malaria transmission. Special emphasis is put on recent insights provided by ecological immunology approaches into the role of mosquito immunity in malaria transmission. Finally, the Section 'Evolutionary assessment of genetically-modified mosquitoes for malaria control' focuses on the contribution of evolutionary concepts in the assessment of novel strategies to control malaria based on the release of transgenic mosquitoes. Specifically, it examines the conditions and consequences of the evolution of mosquito resistance to a lethal genetic construct (population suppression strategy)

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and evolution of parasite resistance to genetically engineered mosquito resistance (population replacement strategy).

Role of mosquitoes in the evolutionary history of *Plasmodium* parasites

With the exception of a single species of lizard malaria, *Plasmodium mexicanum*, which is transmitted by sandflies, all known *Plasmodium* species use mosquitoes (Culicidae) as vectors (Ayala and Lee 1970). While avian and reptilian malaria parasites are transmitted by many mosquito species from several genera (Levine 1988), all known *Plasmodium* species of mammals are transmitted by mosquitoes of the genus *Anopheles*. Recent phylogenetic analyses indicate that *Plasmodium* species infecting mammalian hosts form a well-supported clade associated with anopheline vectors (Figure 1). Although this clade is paraphyletic (it includes parasites of the genus *Hepatocystis*), it suggests that expansion of *Plasmodium* parasites into mammals, including humans, corresponds with specialization into anophelines. This switch from culicine to anopheline mosquitoes, coincident with the expansion into mammals, is believed to have occurred once (Martinsen *et al.* 2008). It is unknown whether specialization into mosquitoes of the genus *Anopheles* is a cause or a consequence of *Plasmodium* expansion into mammals, for a phylogenetic pattern does not enable to infer the mechanism that drove evolution. Nevertheless, the striking associations between the phylogeny of *Plasmodium* parasites and their vertebrate and insect host taxa clearly reveal their three-way phylogenetic interdependence.

The evolutionary origin of *Plasmodium falciparum*, the deadliest human malaria parasite, has been a highly debated topic over the last two decades (Prugnolle *et al.* 2011). What has been controversial is whether *P. falciparum* became a human pathogen as a result of a transfer from ancestral birds, rodents or primates. The most recent evidence supports the origin of *P. falciparum* in gorillas (Liu *et al.* 2010). Although the exact time of the cross-species transmission event is unclear, this finding suggests a relatively recent origin of *P. falciparum*. It supports the view that

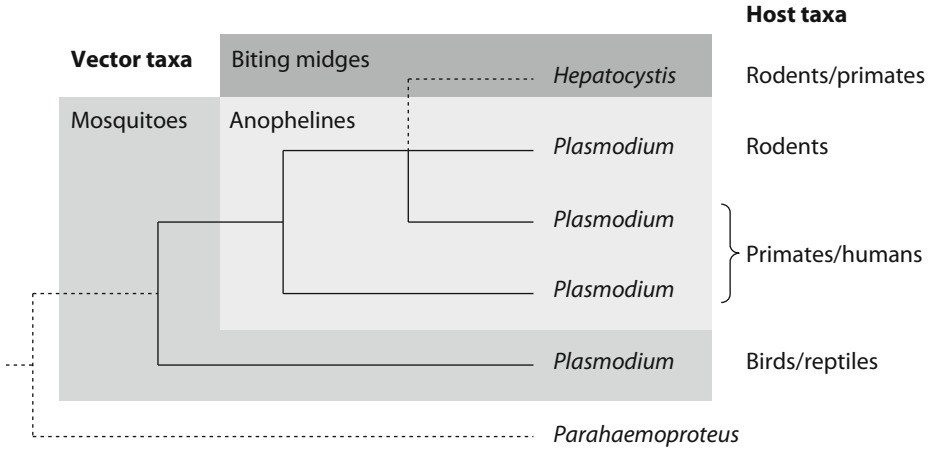

Figure 1. Schematic relationships between the phylogeny of Plasmodium *parasites and their vertebrate host and vector taxa. All clades shown are well supported across different phylogenetic methods. This simple phylogram ignores the only* Plasmodium *species transmitted by sandflies. Adapted from Martinsen* et al. *(2008).*

P. falciparum emerged and expanded in the human population within the last 6,000 years (Rich *et al.* 1998, Volkman *et al.* 2001), coincidental with the emergence of agricultural societies in sub-Saharan Africa. The change in lifestyle from small nomadic groups to larger settled communities would have provided conditions for sustained *P. falciparum* transmission (Hume *et al.* 2003).

In addition, it has been proposed that the expansion of *P. falciparum* in the last few thousand years was facilitated by the speciation process of African mosquito vectors of the *Anopheles gambiae* Giles species complex (Coluzzi *et al.* 2002). The most efficient *P. falciparum* vector species in the complex is *An. gambiae sensu stricto* (*s.s.*). Originally from the African rain forest, this highly anthropophilic species is thought be the product of a speciation process driven by environmental change following the agricultural revolution. Unlike most other anopheline species, *An. gambiae s.s.* mosquitoes typically breed in small, temporary, sunlit freshwater pools. Opening of the vegetation cover by human agriculture, therefore, would have created increased opportunities for larval breeding. Subsequent spread of *An. gambiae s.s.* from the rain forests into savanna areas probably occurred through close association with humans. Interestingly, this scenario is echoed by the current expansion of *Anopheles darlingi* Root, the principal South American malaria vector, following deforestation of the Amazon Basin (Vittor *et al.* 2009).

In Africa, *An. gambiae s.s.* is further diversifying into sympatric ecotypes named M and S (Lawniczak *et al.* 2010). This diversification, probably promoted by adaptation to different larval habitats, may have important consequences for malaria transmission, for the adaptive divergence between incipient species includes that of immune genes conferring resistance to *Plasmodium* (White *et al.* 2010). Another example of diversification is a recently discovered outdoor-resting (exophilic) subgroup of *An. gambiae s.s.*, which lives in sympatry with its indoor-resting (endophilic) counterparts (Riehle *et al.* 2011). This previously unknown population subgroup appears to be highly susceptible to wild *P. falciparum* isolates, and may play a key role in malaria transmission in places where disease control relies primarily on indoor-based vector control measures.

The geographical distribution of anopheline species and populations is clearly an important factor underlying the past and present distribution of human malaria (Hume *et al.* 2003). For example, the relatively inefficient malaria vectors in North Africa (*Anopheles pharoensis* Theobald and *Anopheles sergentii* Theobald) and in the Middle East (*Anopheles pulcherrimus* Theobald) probably constrained the expansion of *P. falciparum* out of Africa (Hume *et al.* 2003). At a more local scale, the genetic structure of vector populations may also play an important role in shaping that of malaria parasites. Combined with the genetic structure of mosquito populations, genetic specificity of compatibility between malaria parasites and their mosquito vectors, both at the species (Billingsley and Sinden 1997) and at the intraspecific level (Christophides *et al.* 2002, Harris *et al.* 2010, Lambrechts *et al.* 2005), promotes opportunities for parasite adaptation to local vector species and populations.

A textbook example of parasite local adaptation to vector species is provided by *Plasmodium vivax*, a human malaria species, in southern Mexico. Two malaria vector species are present at the southern tip of the region between the Pacific coast and the Guatemala border, but with largely non-overlapping geographic distributions. *Anopheles albimanus* Wiedermann is found in the costal region whereas *Anopheles pseudopunctipennis* Theobald occurs in the foothills region. Using microsatellite markers to characterize parasite samples from 98 localities in an area of less than 100 km^2, (Joy *et al.* 2008) found that *P. vivax* consisted of three genetically distinct populations. One population (Figure 2A; white dots) is distributed in the coastal ecoregion, where *An. albimanus* is found, whereas the two other populations (Figure 2A; gray and black dots) are found in the

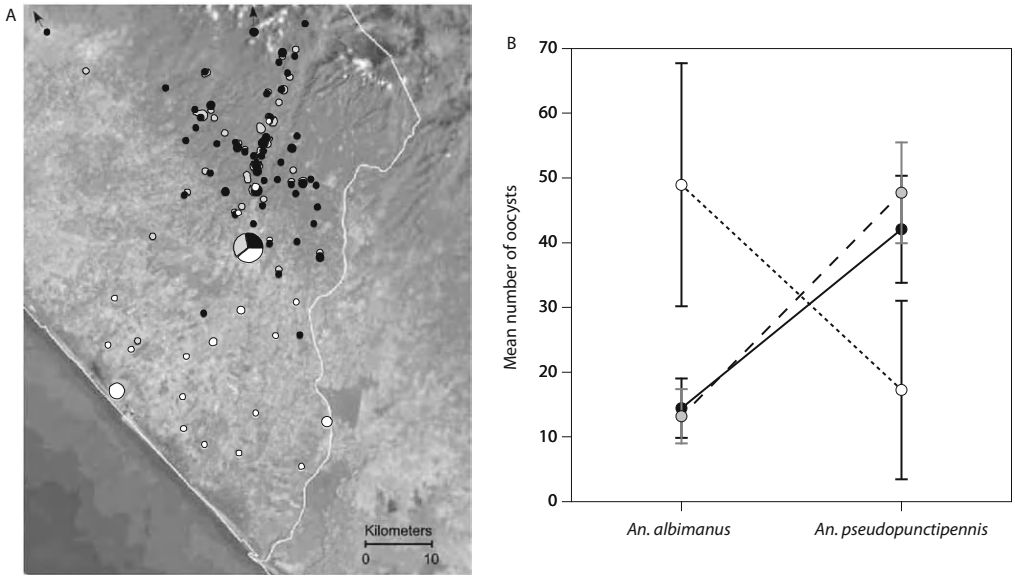

Figure 2. Pattern of adaptation to local vectors in Plasmodium vivax *malaria. (A) Spatial genetic structure of* P. vivax *populations in the Pacific coastal region of southern Chiapas State, Mexico. Each sample was assigned to one of three identified genetically different populations, indicated by different colours. Circle size is proportional to the frequency of the sample, with the exception of the town of Tapachula in the centre. (B) Infection intensities observed in experimental infections of each combination of the three parasite populations and the two mosquito species. Lines represent the parasite genotypes according to the colours of panel A. Vertical bars are 95% confidence intervals. The interaction between mosquito species and parasite genotype is highly statistically significant (*P*<0.0001). Modified, with permission, from (Joy* et al. *2008).*

foothills ecoregion, where *An. pseudopunctipennis* occurs. When they were artificially exposed to the three parasite populations in the laboratory, the two mosquito species were significantly more susceptible to the parasite genotypes encountered in their own ecoregion (Figure 2B). The number of parasite oocysts developing on the mosquitoes' midgut was, on average, about three times higher in sympatric mosquito-parasite combinations than in allopatric pairs. This pattern is suggestive of parasite adaptation to local vectors, indicating that vector distribution can play an important role in generating parasite population structure (Joy *et al.* 2008). Although this process may also act at the mosquito population level within a single vector species, it has, to our knowledge, to date not been documented. In one study that examined the genetic co-structure between *An. gambiae* and *P. falciparum* populations, no pattern was found because of the lack of detectable genetic structure (Prugnolle *et al.* 2008). Because of the recent range expansion of vector and parasite populations, it is very challenging to find neutral markers that are genetically differentiated. In this case, reciprocal experimental infections provide an alternative test of local adaptation (Harris *et al.* 2012).

Understanding the factors shaping the adaptive evolution of *Plasmodium* populations has fundamental implications for public health (Mackinnon and Marsh 2010). Arguably, too little attention has been paid to the role of vectors, relative to humans (e.g. Tanabe *et al.* 2010), in

driving the evolution of malaria parasites at different spatial and temporal scales. In addition to the patterns of genetic compatibility between vector and parasites populations discussed above, variation in host preference among vectors may also have evolutionary and epidemiologically important consequences for malaria transmission (Lyimo and Ferguson 2009). For example, evolutionary invasion analysis accounting for variation in vector anthropophily can help to predict the risk of emergence of *Plasmodium knowlesi*, usually considered a macaque malaria species, in the human population (Yakob *et al.* 2010). More generally, a deeper understanding of macro-evolutionary relationships between *Plasmodium* and their vectors, as well as with their vertebrate hosts, will provide important insights into the history, distribution and incidence of malaria.

Evolutionary ecology of mosquito-malaria interactions

While the precedent section discussed macro-evolutionary patterns among species, this section considers evolutionary forces within single parasite-vector associations, thereby linking micro-evolutionary dynamics with epidemiology.

The epidemiology of malaria is generally summarized by a single metric, the basic reproductive number (R_0), which represents the number of subsequent infections that arise from a single malaria case introduced into an entirely susceptible host population. Mathematically, R_0 can be found from the Ross-MacDonald dynamics (MacDonald 1957) as

$$R_0 = \frac{ma^2e^{-\mu T}}{r\mu} \quad (1)$$

where m is the density of mosquitoes per human host, a is the daily biting rate on humans, μ is the daily mortality rate of mosquitoes, T is the duration in days of the parasite's developmental period within a mosquito (the extrinsic incubation period), and r is the daily recovery rate of infected humans. More precisely, one must distinguish between the biting rate of uninfected mosquitoes on infectious humans, i.e. with gametocytes circulating in their blood (a_1), and the biting rate of mosquitoes that are infectious, i.e. with sporozoites in their salivary glands (a_2). One should also distinguish between the mortality rate of mosquitoes during the parasite's development (μ_1) and the mortality rate once the mosquitoes are infectious (μ_2). Malaria transmission also depends on successful completion of the parasite's development within the mosquito. This is summarized with a parameter, b, which combines the probability that the mosquito becomes infected upon biting a gametocytic human, the probability that the parasite survives through the extrinsic incubation period, and the probability that the bite of a mosquito with sporozoites in its salivary glands infects a human. Thus, Equation 1 can be modified to:

$$R_0 = \frac{ma_1a_2be^{-\mu_1 T}}{r\mu_2} \quad (2)$$

It is striking to note, as MacDonald (1957) realized, that the intensity of malaria transmission is almost exclusively determined by traits related to either the mosquito or the mosquito-parasite interaction. Therefore, the evolutionary pressure for malaria parasites to increase the intensity of transmission will mostly be directed at their interaction with the vectors. Indeed, malaria parasites are able to manipulate most of the parameters in Equation 2 in a way that is expected to increase R_0.

First, infection by the transmissible stages of malaria in humans increases their attractiveness to mosquitoes. Thus, in a semi-natural situation in western Kenya, about twice as many mosquitoes were attracted to children with gametocytes, the infectious stage of malaria in humans, than to children with no detectable infection or with a non-infectious stage of the parasite, but no

gametocytes (Lacroix *et al.* 2005). In other words, the biting rate of uninfected mosquitoes on infectious humans (parameter a_1 in Equation 2) is higher than the biting rate on non-infectious humans, which is epidemiologically irrelevant.

Second, infection by the transmissible stages of malaria in mosquitoes increases their biting rate. In natural situations, mosquitoes with sporozoites, the infectious stage of malaria in the vector, are almost twice as likely to feed on more than one host than mosquitoes without sporozoites (Koella *et al.* 1998). One of the suspected underlying mechanisms is that sporozoites can reduce the activity of apyrase (Rossignol *et al.* 1984), an anticoagulant injected with the mosquito's saliva. As a consequence, mosquitoes take up less blood during the limited time available for a feeding attempt, which increases the probability that the mosquito bites a second time to top up its blood meal (Koella *et al.* 2002). Sporozoites also make mosquitoes more persistent if their blood-feeding attempts are not successful (Anderson *et al.* 1999). Overall, sporozoites increase the biting rate of infectious mosquitoes (parameter a_2 in Equation 2). Note that, as biting is risky (Day and Edman 1984, Edman *et al.* 1984), sporozoites that manipulate the biting rate of mosquitoes also increase their feeding-associated mortality rate (Anderson *et al.* 2000). However, the selective pressure for increased biting overrides the pressure for lower mortality (Koella 1999).

Third, infection by the non-transmissible stages of malaria in mosquitoes decreases their biting rate. Malaria parasites at the oocyst stage do not profit from mosquito biting, because they cannot be transmitted. Rather, any (risky) biting reduces the probability that mosquitoes survive the parasite's extrinsic incubation period. Therefore, oocysts decrease the mosquitoes' biting rate by manipulating them in the opposite way of sporozoites: they decrease persistence if feeding is prevented (Anderson *et al.* 1999), and if feeding is interrupted they decrease the likelihood that the mosquitoes make a second feeding attempt to top up their blood meal (Koella *et al.* 2002). A recent study in Mozambique suggested that malaria parasites may also reduce their vector's biting rate by delaying gonotrophic cycles in *Anopheles funestus* Giles (Charlwood and Tomas 2011).

Thus, four of the parameters in Equation 2 are affected by manipulation of the mosquito's biting behaviour by malaria parasites. Unfortunately, quantitative estimates for malaria-induced changes of these parameters under field conditions are not available. Clearly, however, even small changes of each parameter in Equation 2 will have a large effect on transmission intensity because they act on R_0 in a multiplicative way. Accounting for manipulation of the biting behaviour of mosquitoes by malaria parasites may help to understand the unusually intense malaria transmission in endemic areas. Whereas, for example, the basic reproductive number (a measure of the intensity of transmission) of common childhood diseases ranges within about 10 to 30 (Anderson and May 1991), it can reach more than 1000 for malaria (Smith *et al.* 2007). Importantly, ignoring the evolutionary forces that shape malaria-mosquito relationships would be misleading for entomological studies designed to understand the epidemiology of malaria. For example, mosquito biting rates on gametocytic humans, and biting rates and mortality rates of infected mosquitoes are epidemiologically relevant parameters, whereas biting and mortality rates of mosquitoes in the absence of infection are epidemiologically irrelevant. Measuring the latter would therefore not only underestimate the intensity of malaria transmission, potentially by a considerable amount, but might also underestimate the efforts needed for its control.

Evolutionary forces acting on the mosquito's biting rate also apply to parameter *b* in Equation 2, which represents the ability of malaria parasites to complete their development within the mosquito. As malaria parasites manipulate the mosquito's biting and mortality rates away from values that maximize their reproductive success (Koella 1999), natural selection is expected

to favour mosquitoes that resist infection. In experimental infections, however, a substantial proportion of mosquitoes appear to be susceptible to malaria. For example, *P. falciparum* oocysts developed in 30% of *An. gambiae* mosquitoes exposed to gametocytes in a recent study in East Africa (Menge *et al.* 2006).

To date, most attempts to explain the observed variability of mosquito resistance to malaria have focused on elucidating its genetic basis. Selection procedures demonstrated the existence of a genetic basis for resistance (Collins *et al.* 1986, Vernick *et al.* 1995). Conclusions from these laboratory studies were confirmed with the identification of quantitative trait loci that explain the resistance of field-caught mosquitoes against malaria (Menge *et al.* 2006, Niaré *et al.* 2002, Riehle *et al.* 2006). In parallel, functional studies have revealed the complexity of molecular mechanisms underlying mosquito resistance to malaria. Although resistance also depends on other physiological factors than immunity (Vlachou *et al.* 2005), it is largely governed by a variety of immune responses, which are initiated by pattern recognition receptors regulating downstream effector mechanisms through signal modulation and transduction (for a recent review see Yassine and Osta 2010). Despite considerable progress, it is debatable whether the current understanding, mostly based on molecular studies in laboratory models, of the immune response of anophelines against malaria adequately addresses the complexities of resistance and the intimate co-evolutionary processes between malaria and mosquitoes in nature (Boëte 2009, Boëte 2005, Cohuet *et al.* 2006, Michel *et al.* 2006, see related Chapter by Pike *et al.* in this book).

One complicating factor is that environmental variation can have a considerable effect on the expression of resistance, including its genetic basis. For example, symbiotic gut bacteria (Dong *et al.* 2009, Meister *et al.* 2009) and parasitic microsporidia (Bargielowski and Koella 2009) strongly influence the prevalence of *Plasmodium* infection in mosquitoes. In addition, the concentration of sugar fed to mosquitoes after infection by malaria affects not only infection success, but also the differences among isofemale lines, i.e. the extent to which genes contribute to the observed variation of resistance (Hurd 2007, Lambrechts *et al.* 2006a).

More importantly, the interaction between malaria and mosquitoes is not governed by the mosquito's genes alone, but by the interaction between the mosquito's and the parasite's genes (Harris *et al.* 2010, Lambrechts *et al.* 2005), as is the case for many host-parasite interactions (Lambrechts *et al.* 2006b). Thus, a particular gene variant may make mosquitoes (partly) resistant against some malaria genotypes but not to others, while other gene variants make other mosquitoes resistant to other malaria genotypes. As a consequence, it is unlikely that a single resistance gene variant can confer resistance against all malaria genotypes, and that a single malaria genotype can overcome all mosquito resistance gene variants. Such a situation leads to either of two co-evolutionary outcomes: a stable coexistence of multiple resistance variants, or continuous cycling of the different variants (e.g. Leonard 1994). Due to such genotype-by-genotype interactions and their evolutionary dynamics, it is not very surprising that attempts to find adaptive signatures in anti-*Plasmodium* mosquito immune genes, which test for positive, directional selection on gene variants, were inconclusive so far (Obbard *et al.* 2008, Parmakelis *et al.* 2008, Slotman *et al.* 2007).

The realization that studies of laboratory systems provide limited insights into the intimately co-evolved systems of malaria and mosquitoes in nature is bringing molecular biologists and evolutionary ecologists together to study the variation of mosquito resistance against malaria in their natural ecological and evolutionary context, an emerging field referred to as the 'ecological immunology' of mosquito-malaria interactions (Tripet *et al.* 2008). This approach focuses on

questions of function and adaptation, with less emphasis on the molecular mechanisms of immunity and resistance (Schmid-Hempel 2005). At the centre of this approach is the idea that a balance between evolutionary costs and benefits determines the level of resistance in a population. On the one hand, as discussed above, malaria parasites manipulate the mosquito's biting rate and mortality away from the mosquito's optimum. Malaria infection can also directly affect the mosquito's physiology, reducing its fecundity and survival (reviewed in Tripet *et al.* 2008). Because of such fitness costs associated with malaria infection, resistance is clearly beneficial to the mosquito. On the other hand, the mosquito's immune response and resistance also involve costs, which are thought to result in part from physiological trade-offs due to shared pathways between immune responses and other physiological functions (Tripet *et al.* 2008). Such an evolutionary trade-off was demonstrated by the observation that mosquitoes selected for rapid development have a weaker melanisation immune response than those selected for slow development (Koella and Boëte 2002). Because of the genetic correlation between the two traits, any evolutionary change in one trait would be associated with changes in the other trait. Overall, it is expected that evolution will increase resistance (resulting from increased immune function) only to the level where its benefit is balanced by its cost.

That evolution balances costs and benefits may be the main mechanism for the maintenance of susceptibility in natural populations (Boëte and Koella 2003, Tripet *et al.* 2008, see Chapter 2 in this book by Pike *et al.*). This evolutionary perspective may also help to understand why the number of malaria oocysts observed in naturally infected mosquitoes is low. Indeed, most mosquitoes infected with *P. falciparum* in natural populations have a single oocyst of the parasite (e.g. Lyimo and Koella 1992), whereas in laboratory systems they harbour many more. One possible explanation is that evolution has led to a balance between the disadvantages and advantages of resistance. On the one hand, weak resistance would lead to high oocyst loads, which would result in substantial damage. For example, the ability to manipulate biting rate increases with parasite load. Re-analysis of the data in Koella *et al.* (1998) shows that whereas about 22% of the mosquitoes infected with sporozoites bit more than one person if their sporozoite load was lower than the median, about 36% did so if their sporozoite load was greater than the median. On the other hand, a very effective immune response leading to complete parasite clearance is likely to be very costly, both in terms of expenditure of energy and in terms of the production of toxic compounds such as melanin. Therefore, an intermediate level of resistance, enabling low-level infection with moderate pathogenic effects, may be optimal for the mosquito. While some preliminary data support this idea (Boëte *et al.* 2004), the idea has not been formally tested.

This section emphasized that fully understanding the epidemiology of malaria and the biology of its vectors requires careful consideration of the micro-evolutionary relationships between mosquitoes and malaria parasites. Although the influence of evolutionary forces on the vectorial capacity of mosquitoes for malaria has been clearly demonstrated by proof-of-principle, qualitative experiments, much work remain to be done to turn predictions and speculations into quantitative assessments. Understanding how the co-evolution of malaria and mosquitoes affects the epidemiology of malaria and the pattern of resistance of mosquitoes in nature remains a major challenge.

Evolutionary assessment of genetically-modified mosquitoes for malaria control

This last section is intended to illustrate how the approach of evolutionary ecology described in the previous section can be used to assess the feasibility of a malaria control strategy. As it is beyond the scope of this chapter (and current knowledge) to give a detailed and quantitative

prediction, we restrict ourselves to a few selected studies describing critical aspects rather than giving an extensive review of the relevant literature. In the prospect of releasing genetically-modified mosquitoes to control or eliminate malaria, it is crucial to understand the potential evolutionary trajectory and evaluate the impact on the incidence of disease. More details on this topic are available in several earlier publications (Boëte and Koella 2002, Koella and Boëte 2003, Koella and Zaghloul 2008).

Failure of existing malaria control methods have stimulated research to develop novel, innovative strategies. In the last two decades, breakthroughs in the molecular genetics of mosquitoes have provided the groundwork for implementing vector control strategies based on the release of genetically-modified mosquitoes (Alphey *et al.* 2002, Catteruccia 2007, Christophides 2005). These strategies aim at either replacing the existing wild vector population with engineered vectors that are refractory to the pathogen (population replacement) (Nirmala and James 2003) or eliminating the wild vector population using a genetic system that reduces reproductive capacity (population suppression) (Alphey *et al.* 2008). In the case of a population replacement strategy, the task is two-fold. It consists of (1) genetically engineering mosquitoes that are refractory to the pathogen and (2) driving the genetic construct conferring refractoriness to fixation into the wild target population. Although transgenic mosquito lines impaired in malaria transmission have been created in the laboratory (Corby-Harris *et al.* 2010, Ito *et al.* 2002), much remains to be done before these proofs-of-principle are converted into a feasible malaria control tool (Hill *et al.* 2005). One of the major challenges ahead is optimization of genetic drive systems to deliver the refractory transgenes into wild vector populations (Marshall and Taylor 2009). Naturally occurring selfish genetic elements, such as transposons, meiotic drive genes, endosymbiotic bacteria or homing endonuclease genes, are promising candidates to develop such genetic drive systems (Sinkins and Gould 2006). A synthetic selfish genetic element has been shown to drive population replacement in *Drosophila* (Chen *et al.* 2007), and very recently in *An. gambiae* (Windbichler *et al.* 2011).

By contrast, genetic strategies of vector population suppression generally do not require such a gene drive system because they rely on the repeated mass releases of transgenic sterile insects (Alphey *et al.* 2008). The disabled insects mate with wild individuals in the target population, thereby reducing its reproductive output, and potentially resulting in the subsequent collapse of the wild population if sufficient numbers of sexually competitive transgenic insects are released. The potential of this strategy has been recently supported by the successful elimination of caged populations of the dengue virus vector *Aedes aegypti* L. under semi-field conditions (Wise de Valdez *et al.* 2011), and during a recent open-field trial on the Cayman Islands (Harris *et al.* 2011). Thus, despite reduced mating competitiveness and smaller adult body size of genetically-modified mosquitoes compared to their wild type counterparts (Bargielowski *et al.* 2011a,b), frequent releases can compensate for the fitness costs.

There are at least three critical aspects that are often overlooked in the development of malaria control strategies based on genetically-modified mosquitoes. First, in the case of a population replacement strategy, the epidemiological feed back on the population genetic process influences the conditions allowing fixation of the trait of interest. The spread of an allele conferring refractoriness to malaria in a mosquito population was examined by a model combining population genetics with epidemiology (Boëte and Koella 2002). This model demonstrated that because the change in malaria prevalence in the human population feeds backs onto the mosquito fitness, which depends in part on the evolutionary costs and benefits of refractoriness, fixation of refractoriness requires a genetic drive mechanism that increases the frequency of inheritance of the genetic element compared to regular Mendelian inheritance. Although refractoriness confers

a clear evolutionary advantage to the mosquitoes when parasite prevalence is high (Marrelli *et al.* 2007), this advantage decreases progressively when transmission and parasite prevalence are decreasing due to the spread of refractoriness (Lambrechts *et al.* 2008). Luckily, a moderate efficacy of the drive mechanism is enough to spread a refractoriness allele to fixation in most situations (Boëte and Koella 2002).

Second, the evolutionary response of the parasite can significantly threaten the effectiveness of the intervention outcome. Parasites are often able to evade or suppress the defence mechanisms of their hosts, including *Plasmodium* parasites in their mosquito vectors (Boëte *et al.* 2004, Lambrechts *et al.* 2007). A co-evolutionary model predicted that the optimal level of malaria parasite investment into evasion or suppression mechanisms depends on the relative investment into defence mechanisms of the mosquito (Koella and Boëte 2003). Thus, in the case of a vector population replacement strategy, introducing a genetic construct conferring refractoriness to malaria infection may alter this co-evolutionary balance towards greater investment of the parasite into evading or suppressing the refractoriness mechanism. This would result in a decreased efficacy of refractoriness, which was showed to dramatically reduce the impact of the control programme on malaria prevalence in the human population (Boëte and Koella 2002, Koella and Zaghloul 2008). One way of avoiding, or at least delaying, this co-evolutionary response may be to transform mosquitoes with mechanisms of refractoriness that are not found in natural systems. It would be less likely that standing genetic variation for suppressing or evading the refractory mechanism is already present in the parasite population. Therefore, the co-evolutionary response would be delayed until the necessary mutations have arisen.

Third, in the case of a population suppression strategy, it is important to carefully consider the evolutionary response of the wild vector population. For example, evolution of resistance to the lethal mechanism is a potential threat to population suppression strategies based on the release of mosquitoes carrying a dominant, repressible, lethal genetic construct. Mathematical modelling showed that although resistant alleles are unable to spread to fixation, they can become more common than the alternative susceptible allele and therefore have a detrimental impact on the release program (Alphey *et al.* 2011).

In conclusion, there is much to gain by including an evolutionary approach to assess the feasibility of using genetically-modified mosquitoes for malaria control. Not only evolutionary mathematical modelling, but also experimental evolutionary ecology, can provide important insights into the potential success of these strategies (Boëte and Koella 2003).

Conclusions and perspectives

The evolutionary forces underlying the vectorial capacity of anopheline mosquitoes for malaria parasites are still poorly understood. One of the greatest challenges for future research is to obtain quantitative, epidemiologically relevant estimates of some of the critical parameters underlying malaria transmission. This will only be achieved by studying natural systems of *Anopheles-Plasmodium* interactions. At least in studies designed with the goal of understanding malaria epidemiology, efforts must be made so that experiments emulate more closely the natural conditions that are meaningful for parasite transmission. For example, whether malaria parasites kill their vectors has been largely debated (Ferguson and Read 2002), but most of the studies on the effect of malaria infection on mosquito survival ignore feeding-associated mortality, which is likely to account for most of the mortality in nature. A related challenge is to account for the influence of manifold abiotic factors on mosquito-parasite interactions and their implications

for malaria transmission. For example, the impact of short-term temperature fluctuations on the development of malaria parasites in mosquitoes has recently been revealed, either speeding up or slowing down processes depending on the mean temperature (Paaijmans *et al.* 2010). This highlights the need for a better understanding of the mechanistic link between environmental heterogeneity and vectorial capacity. More generally, deciphering mosquito-parasite dynamics and co-evolution will require moving from laboratory conditions to more realistic systems.

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Species-specific interactions

7. Tick – *Borrelia* interactions: burden or benefit?

Fedor Gassner and Nienke Hartemink

Abstract

A key factor in the success of parasites is the ability to move between hosts. Some parasites make use of an intermediate arthropod host to move between their primary hosts. Several examples exist where such parasites manipulate their intermediate host to enhance their transmission, but examples for ticks are scarce. In this chapter, we describe how *Borrelia burgdorferi sensu lato*, the causative agent of Lyme borreliosis, is associated with changes in the behaviour, physiology and survival of *Ixodes* ticks. Such changes can lead to more effective host finding for the tick and better colonisation of new hosts by *Borrelia*. We discuss how these changes may lead to an increased transmission (risk) of *Borrelia*. A next-generation matrix approach is applied to model potential effects of increased tick survival on the basic reproduction number R_0 of *Borrelia*. Using this approach, we show that *Borrelia*-associated increased survival of ticks can have a profound effect on the circulation of spirochaetes, and hence on Lyme borreliosis risk. Future studies would ideally resolve the mechanisms behind the described changes, and establish experimentally whether *Borrelia* can enhance its circulation between hosts.

Keywords: behaviour, *Borrelia*, *Ixodes*, Lyme, manipulation, R_0, tick

Introduction

A critical step in the life cycle of all parasites is to move from one host to the next. Many parasites can readily disperse between hosts without the help of a third biological party. Such dispersal can be passive, for example bacteria, fungi and viruses that become airborne as single or clumped individuals, or adhered to moist or dust aerosols. Other parasites disperse actively by use of flagella or muscular propulsion. More complex forms of parasitic dispersal occur when a parasite uses another intermediate organism for their dispersal. Often, this intermediate organism is an arthropod, which is then referred to as a vector.

Many examples exist of parasites that modify their vector to for their own benefit (see for reviews Dobson 1988, Hurd 2003, Lefèvre and Thomas 2008, Schaub 2006). For example, mosquito host-searching and blood-feeding behaviour can be affected by malaria parasites (Koella and Packer 1996, Koella *et al.* 2002) and sandflies show altered feeding behaviour when they are infected with *Leishmania* parasites (Ready 2008, Rogers and Bates 2007). Parasite-driven change of vector behaviour can result in an increased transmission rate of the pathogen, and, therefore, increased human health risks.

In contrast to the numerous examples of parasite-mediated changes in insects, few examples exist for the class of Arachnida. It is unlikely that such mechanisms are biased for insects alone. Indeed, some examples exist for effects of bacteria that alter mite behaviour (Schütte and Dicke 2008, Schütte *et al.* 2006). Despite the medical and veterinary importance of tick-borne diseases, very few studies have focussed on potential pathogen-mediated changes in the biology of ticks. In this chapter, we describe how specific interactions enable the transmission of the spirochete *Borrelia burgdorferi sensu lato*, the causative agent of Lyme borreliosis, by the sheep tick *Ixodes ricinus* L. (Acari: Ixodidae) between hosts.

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Among blood-sucking arthropod vectors that transmit pathogenic agents, ticks hold the reputation of being able to transmit the greatest diversity of pathogens (Anderson and Magnarelli 2008). Apart from *Borrelia* species, the sheep tick *I. ricinus,* with its distribution throughout Europe, western Asia and North Africa, and its closely related American (*Ixodes scapularis* Say and *Ixodes pacificus* Cooley & Kohls) and Asian (*Ixodes persulcatus* Schulze) counterparts are known to transmit an array of other pathogens. The most important human pathogens are tick-borne encephalitis virus (Europe and Asia) and *Anaplasma*, *Rickettsia* and *Babesia* species. Over the past decades, transmission of *B. burgdorferi s.l.* by several species of hard ticks within the *Ixodes* genus has received increasing attention. *Borrelia* species are known to cause Lyme borreliosis in humans as well as livestock and pets, and are prevalent across the northern hemisphere.

Although decreasing in some areas, the incidence of Lyme borreliosis seems to increase throughout most of its range (Heyman *et al.* 2010, Smith and Takkinen 2006). Part of the increase may be attributed to increased awareness and improved diagnostic tools. In addition, changes in climatic conditions and land use can strongly affect ticks, their hosts, their habitat and pathogen transmission dynamics, leading to spatial as well as temporal variation in (pathogen infected) tick abundance. Human behaviour also affects the intensity of contact with ticks carrying pathogens, for example by increasing activity in habitats with high tick densities (Lane *et al.* 2004).

Apart from the established pathogens, ticks harbour a great diversity of other, mostly undescribed, micro-organisms (Halos *et al.* 2006, Stanek 2009, Van Overbeek *et al.* 2008). Such organisms may play a role in symbiotic or pathogenic relations with the tick. Their pathogenic traits, their interactions with other pathogens, and their impact on tick biology largely remain to be studied.

The ecology of Lyme borreliosis

The ecology that underlies Lyme borreliosis includes seven interrelated components that can all be affected by climate and human influence (Figure 1). Covering all components, human influence, such as host or habitat alteration, and the climate, especially dry or cold conditions, strongly affect the ecology of Lyme borreliosis. For example, *I. ricinus* is very susceptible to desiccation, preferring a relative humidity >80%, and will become active at temperatures above ~4 °C (Lees 1946, 1948, Perret *et al.* 2000, Sonenshine 1991). Hosts form a key component in the ecology of Lyme borreliosis by providing ticks with a blood meal, by acting as reservoir for *Borrelia* and by providing dispersion for ticks as well as pathogens. Such hosts are closely associated with habitat characteristics, and most host species are subject to predation. In turn, these predators may act as host for the tick. A classical example may be the role of wood mice (*Apodemus sylvaticus* L.) as reservoir for *Borrelia* species and blood host for immature *I. ricinus* (Matuschka *et al.* 1992). These mice are associated with forested habitats, a habitat shared by the red fox (*Vulpes vulpes* L.), a common predator for small rodents. In turn, the red fox can act as blood host to ticks (Gern 2008). Consequently, predator prey population fluctuations can have profound effects on tick and *Borrelia* population dynamics. Of course, other alternative blood hosts will also play a role in most habitats and may either amplify or reduce local risk for Lyme borreliosis, and thereby add to the complexity of Lyme borreliosis ecology.

Ticks are also strongly influenced by soil and vegetation characteristics, two factors that are in turn strongly interrelated. Vegetation may attract hosts, for example by providing shelter or food, but can also provide a suitable microclimate for tick survival. Soil characteristics in turn affect tick abundance, since ticks depend on a suitable litter layer for shelter, rehydration during dry

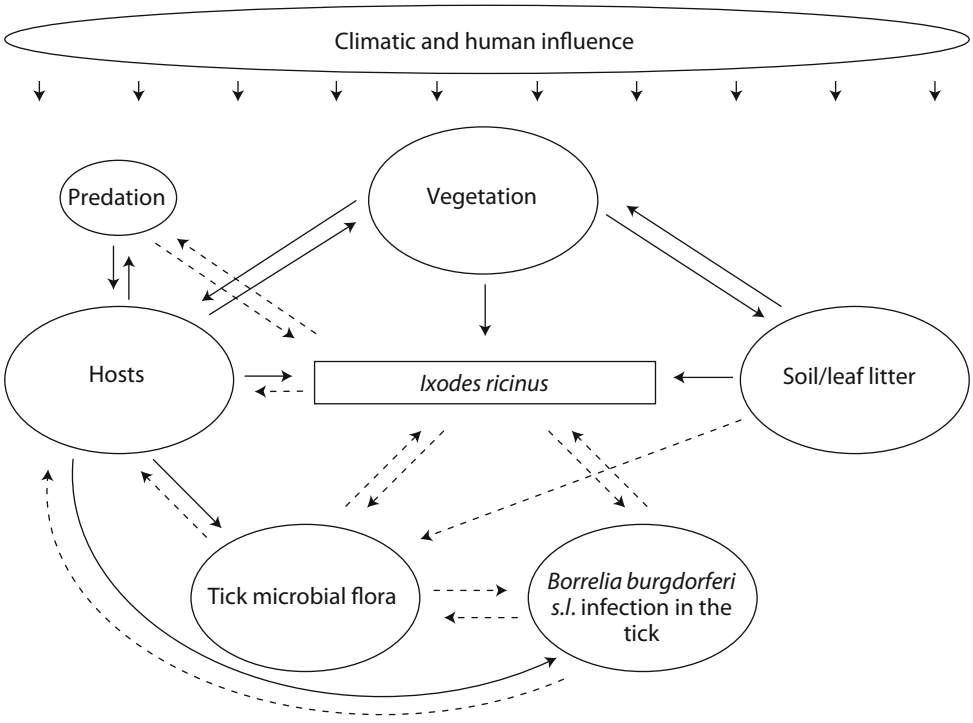

Figure 1. Schematic representation of the ecology of Ixodes ricinus *ticks. Solid lines indicate pathways that have been extensively studied, dotted lines indicate interactions that are unstudied or scarcely studied (Gassner, 2010).*

periods and as a refuge during diapause events. Indeed, abundance of *Ixodes* ticks is correlated to properties of the litter layer (Eisen *et al.* 2004, Gassner *et al.* 2011).

The range of micro-organisms and viruses that can be found in ticks are either transmitted from hosts during blood meals, or transmitted vertically from mother to eggs and trans-stadially from egg to larva, from larva to nymph and from nymph to adult. Other transmission mechanisms have not been described, but some micro-organisms could potentially be picked up from the soil. Microorganisms could potentially invade the tick body along with other penetrating parasites such as nematodes or parasitic wasps (Plantard *et al.* 2012, Tijsse-Klasen *et al.* 2011). Interactions between various viruses, nematodes, bacteria and protozoa inside ticks remain largely unstudied, but simultaneous transmission of two pathogens to a host may be beneficial for either pathogen.

Perhaps the most essential determinant in the ecology of Lyme borreliosis is the tick's succes of finding a host and thereby transmit and/or pick up *Borreliae*. This depends not only on host density, but also on the activity of the tick. Any mechanism that may enhance tick activity may therefore contribute to the ability of *Borrelia* to invade a new host, and therefore contribute to an increased basic reproduction number R_0.

Biology of Ixodes ricinus *and* Borrelia burgdorferi sensu lato

Given the fact that suitable environmental conditions and host availability are present, the tick life cycle can be completed in three years under natural conditions (Randolph *et al.* 2002), but under unsuitable conditions, may be stretched to six years (Figure 2). After hatching or moulting, newly-emerged ticks start questing (i.e. searching for a host in the vegetation) when environmental conditions are suitable (Lees 1948, Perret *et al.* 2000, Randolph and Storey 1999). During questing, ticks stay in ambush in the undergrowth to grab hold of passing hosts. While active, the forelegs are protruded in order to sense host presence using the ticks Haller's organ, which contain olfactory sensilla. During periods of desiccating conditions, ticks descend close to or in the litter layer to restore their water balance by taking up water vapour from the air. The tick utilizes a variety of vertebrate hosts for its three blood meals, which are needed for successive moulting from larva to nymph, from nymph to adult and for the development of eggs by adult females (Figure 2). In general, larvae stay close to the ground, where they attach to small hosts such as rodents and song birds, as well as occasionally to larger hosts (Vor *et al.* 2010). Nymphs have a much wider

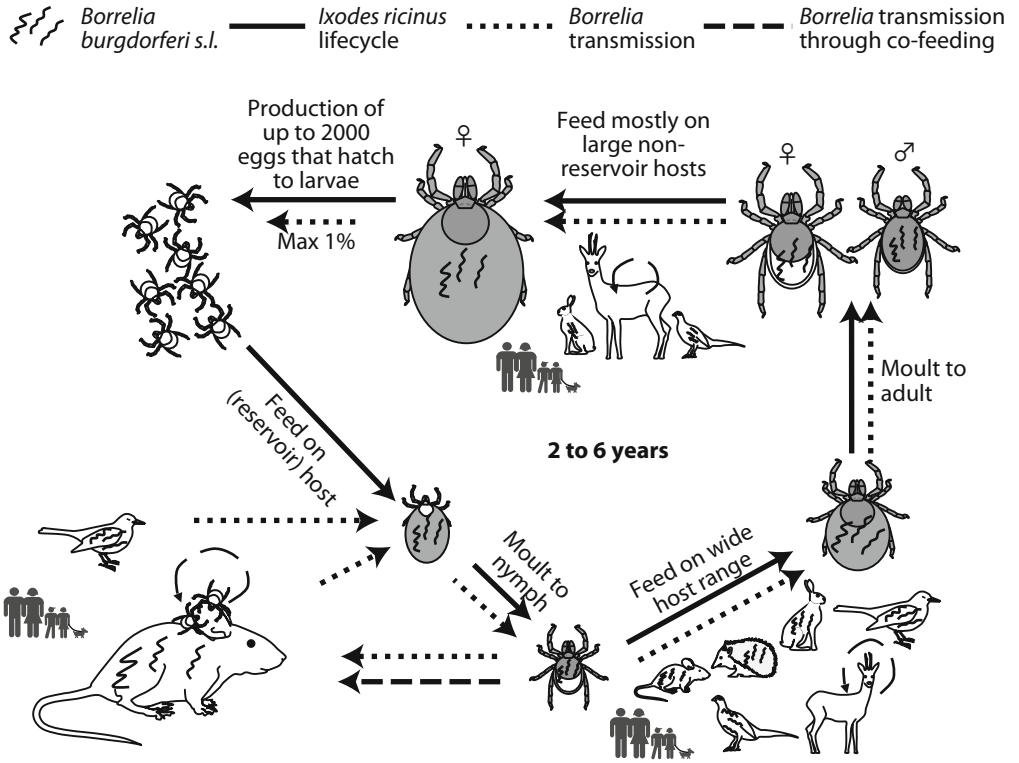

Figure 2. Life cycle of Ixodes ricinus *in relation to the natural circulation of* Borrelia burgdorferi s.l. *among several hosts. Note that all tick life stages feed on humans next to their natural hosts. The hosts that are drawn with* Borrelia *represent reservoir hosts that can re-infect ticks through a systemic infection. The depicted hosts represent the diversity of hosts, the true host range may be larger in nature (Modified with permission after Gassner 2010).*

host preference and tend to use questing points from ground level up to approximately 50 cm. Adult females preferably feed on larger hosts such as deer, while the adult males mate several females either in the vegetation or on hosts, without taking substantial blood meals. The adults tend to climb higher into the vegetation, from ground level to approximately 150 cm (Mejlon and Jaenson 1997).

Borrelia species can be acquired by the tick by a blood meal on a reservoir-competent host, and can be maintained trans-stadially and passed to the next host during the second and third blood meal. In turn, vertebrate hosts can become (systemically) infected by *Borrelia* species through the bite of an infected tick (Figure 2). These transmission processes, from tick to host and vice versa, are referred to as systemic transmission. A second route is non-systemic or co-feeding transmission: ticks acquire the pathogen from infected ticks feeding at sites in their immediate vicinity without the need for a systemic infection in the host (Gern and Rais 1996, Ogden 1997, Patrican 1997, Richter *et al.* 2002). The quantitative contribution of non-systemic transmission to the maintenance of Lyme borreliosis spirochaetes through common reservoirs such as rodents is subject to debate (Randolph and Gern 2003, Richter *et al.* 2003), but co-feeding transmission can facilitate tick to tick transmission in otherwise reservoir incompetent hosts such as sheep (Ogden, 1997). The role of transovarial transmission –from an infected female adult to her offspring – for *Borrelia* is not clear. A fraction of (unfed) larva in the field is infected with *Borrelia*, suggesting that they have been infected via this route (Rauter and Hartung 2005). However, in laboratory experiments eggs failed to inherit *Borrelia* (*afzelii*) from their infected mother (Matuschka *et al.* 1998). Whether the infected larvae in the field are a result of partial feeding (e.g. as a result of host death, damaging immune responses to the tick bite, or physical disturbance by the host), transovarial transmission, lack of *Borrelia* detection specificity, or differences between *Borrelia* genospecies remains to be investigated.

Although mice and songbirds are considered to be the primary reservoir of *Borrelia* species, other species of mammals, birds and lizards have also been identified to be able to transmit *Borreliae* to feeding ticks (reviewed in Piesman and Gern 2008). Globally, the genus *Borrelia* consists of at least 16 genospecies (Margos *et al.* 2011), commonly named *Borrelia burgdorferi sensu lato*. In Europe, several *Borrelia* species have been associated with specific hosts, where *Borrelia* species infections are confined mostly by the host immune system. Of the most common species, *B. afzelii* and *Borrelia bavariensis* are associated with rodents. *Borrelia valaisiana* and *Borrelia garinii* have been associated with birds and *Borrelia lusitaniae* has been associated with lizards (Hanincová *et al.* 2003a,b, Humair *et al.* 1999).

Borrelia – tick interactions

Travelling through various environments

Borrelia bacteria may find various environments during the course of their transmission, which may be accompanied by specific gene expression to adapt to these environments. Part of this adaptation is regulated through a range of *Borrelia* outer surface proteins (Osp's). Inside the reservoir host body, *Borreliae* may reside in various tissues such as heart, joints, brains and skin, or be transported in the host blood circulation (Barbour *et al.* 2009, Gray *et al.* 1999, Hanincová *et al.* 2003a, Kurtenbach *et al.* 1998, Schwan *et al.* 1988). Inside these environments, the spirochaetes are continuously exposed to various components in the host immune system, while causing a persistent infection, which can last months in the case of rodents (Gern *et al.* 1994). Once a tick attaches to an infected and reservoir-competent host, the *Borreliae* can be ingested, ending up in

the tick midgut along with ingested host blood. Here, they will remain during the remaining blood meal of the tick, attach to the midgut lumen, and be exposed to the immune as well as digestive system of the tick. After engorgement by the tick, the *Borreliae* face the challenge of surviving during the tick transstadial moulting process, which can be followed by prolonged periods of diapause, questing or quiescence. Although the physiology of *Borrelia* and the tick at the time of the blood meal have been extensively studied, little is known about the physiology of *Borrelia* during the period between blood meals.

Once the tick attaches to a new host to take a blood meal, *Borreliae* will detach from the midgut lumen and proliferate, while migrating through the tick haemolymph to the salivary glands. The duration of this process differs per tick species and *Borrelia* species; in *B. burgdorferi sensu stricto* infected *I. scapularis* it was found to take place within 36-48 hours (De Silva and Fikrig 1995). However, for *B. afzelii* in *I. ricinus*, dissemination from the midgut to the salivary glands is much faster and can result in *Borrelia* transmission within 24 hours (Crippa *et al.* 2002). Therefore, it is generally advised to remove attached ticks within 24 hours after attachment, but the sooner the better. The *Borrelia* OspC is involved in the migration (Schwan and Piesman 2002), but once arrived in the salivary glands, *Borreliae* will be actively injected into the new host along with the tick saliva.

In summary, *Borrelia* is exposed to different within-host environments, as well as at least three different within-tick environments (i.e. midgut lumen, haemolymph and salivary glands). Specific tick- as well as *Borrelia*-induced interactions during the tick-borne phase of *Borrelia* will be described below, with an emphasis on saliva assisted transmission, *Borrelia*-associated behavioural changes in the tick and *Borrelia*-associated increase in tick survival. For reviews on the first topic see Mannelli *et al.* (2012) and Schuijt *et al.* (2011).

Tick – Borrelia *interaction during feeding: saliva assisted transmission*

The feeding stage of *I. ricinus* takes at least three days for larvae, to up to 7 to 10 days for adult females. Throughout the feeding period, the tick salivary glands secrete a wide array of pharmacologically active molecules. The primary function of these molecules is to suppress blood clotting, inflammatory responses and immune responses in the host. Suppressing these processes in the host, as well as in ingested blood, is highly important due to the long feeding period of the tick, which is among the highest of blood-feeding ectoparasites. Some of these salivary products are known to aid pathogens in their transmission, a process referred to as saliva-assisted transmission (SAT). SAT can be direct, where salivary molecules interact with the pathogen or the host in favour of the pathogen and/or the tick. Indirect SAT can refer to non-systemic transmission through the co-feeding process described above, where pathogens are exchanged in the direct vicinity of the tick bite, without the need of a systemic infection. Several tick-borne pathogens have been associated with SAT in *I. ricinus*, including tick-borne encephalitis virus, *Francisella tularensis, B. lusitaniae* and *B. afzelii* (Nuttall and Labuda 2008). In North America, SAT has been described for *B. burgdorferi sensu stricto* in *I. scapularis* ticks (Ramamoorthi *et al.* 2005, Zeidner *et al.* 2002).

A well-described mechanism of SAT in *I. ricinus* and *I. scapularis* is the excretion of the Salp15 protein (Hovius *et al.* 2008, Ramamoorthi *et al.* 2005, Schuijt *et al.* 2008), which has also been described in *I. persulcatus* and *I. pacificus* (Hojgaard *et al.* 2009). Salp15 suppresses a T-cell mediated immune response in the host, and binds to the *Borrelia* OspC. This binding provides *Borrelia* with a tick-produced protective coat against host antibodies. This mechanism has been experimentally established for several, but not yet all, relevant *Borrelia* genospecies (Hovius *et al.* 2008).

Interestingly, genes encoding for Salp15 are more strongly upregulated during blood feeding of *Borrelia*-infected ticks compared to uninfected ticks (Ramamoorthi *et al.* 2005). In this way, *Borrelia*-mediated changes in the tick physiology may enhance survival of *Borrelia*.

Tick – Borrelia *interactions during non-feeding periods*

Borrelia is generally assumed to remain bound to the tick midgut lumen by outer surface proteins (Osp's). Although many processes, molecules and gene expression profiles have been described for tick-*Borrelia* interactions during the blood-feeding stage, very little is known about tick-*Borrelia* interactions in between blood meals, i.e. in unengorged ticks that have moulted after their blood meal and are either questing in the undergrowth or inactive in the litter layer. It is generally assumed that *Borrelia* remains molecularly bound to the midgut lumen of flat ticks using OspA, and possibly OspB, which bind to the tick receptor molecule TROSPA (Pal *et al.* 2004).

To date, several interactions between ticks and *Borrelia* in the non-feeding phase have been reported, including *Borrelia* associated changes in tick behaviour, phenotype, development or response to abiotic factors.

Borrelia-*induced behavioural modifications*

Effects of infection with *Borrelia* on *I. ricinus* have rarely been examined in the laboratory. Lecfort and Durden (1996) showed some effects of infection with *B. burgdorferi s.s.* on the behaviour of laboratory-reared *I. scapularis*. They found overall decreased activity in infected adult ticks, whereas infected nymphs showed increased phototaxis and increased activity on the vertical surfaces during their experiments. Increased activity in nymphs would potentially increase the transmission potential of *Borrelia*. Nymphs are, in case of absence or low rate of transovarial transmission, the most important source of infection for naïve rodents, which are in turn needed for infection of naïve larvae. As adult ticks are less likely to feed on reservoir-competent hosts, behavioural changes in this life stage are less useful for *Borrelia* species transmission. Alekseev *et al.* (2000) found reduced activity in infected individuals for both *I. ricinus* and *I. persulcatus* during 3 min behavioural assays, whereas increased activity was observed in *Borrelia*-infected adult *I. persulcatus* that also showed morphological anomalies. These contradictory results are likely to be influenced by experimental setup (i.e. strong influence of the observer on tick behaviour) and the short time span of the experiments. Later on, Perret (2003) showed that field collected *Borrelia*-infected, *I. ricinus* nymphs display increased activity over prolonged periods of time under desiccating conditions compared to uninfected nymphs. In a recent experiment, velocity and the duration of walking activities were elevated in wild-captured as well as laboratory-reared *B. afzelii*-infected versus uninfected *I. ricinus* nymphs (Gassner 2010). In theory, increased tick activity can substantially increase the host contact rate, especially with ground-dwelling rodents. This behaviour may lead to increased survival of ticks, as well as increased *Borrelia* transmission (Hurd 2009). However, future experimental evidence is required to elucidate this behaviour.

Field observations of behavioural changes in *Borrelia*-infected members of the *I. ricinus* species complex are rare. For example, more *Borrelia*-infected *I. pacificus* ticks were found on vertical objects such as tree trunks compared to the surrounding litter (Lane *et al.* 2007). In Germany, adult *I. ricinus* females attached to human volunteers were more frequently infected with *Borrelia* species compared to the infection prevalence of female ticks collected by blanket dragging in the same area where the volunteers had walked (Faulde and Robbins 2008). Although field evidence

for *Borrelia*-driven behavioural changes in *I. ricinus* is mostly indirect, it supports the observations of increased activity due to *Borrelia*-infections in the lab.

Next to increased host-finding chance, the increased walking speed of infected ticks – in the study of Gassner (2010) there was an ~10% increase in the mean velocity – can cause ticks to find a suitable attachment place more efficiently on the host, and thereby lower the chance of detection or falling off prior to attachment.

Hence, we argue that *Borrelia*-induced behavioural changes can increase *Borrelia* transmission in nature. Additionally, the risk of a bite from an infected tick may be higher than the risk of a bite of an uninfected tick under similar densities of both groups in the field. The potential impact of *Borrelia*-associated behavioural changes on the R_0 of *Borrelia* will be discussed later in this chapter.

Borrelia-*associated changes in response to microclimate*

Next to *Borrelia*-associated increased activity of ticks, other recent observations indicate that *Borrelia*-infected *I. ricinus* ticks are better equipped to withstand desiccation conditions. Hermann and Gern (2010) found increased resistance to desiccation in nymphal as well as adult *Borrelia*-infected *I. ricinus*, compared to uninfected nymphs and adults. Here, the load of *Borrelia* spirochetes was quantified, indicating that a load of over 160,000 spirochetes decreases adult survival, whereas increased desiccation resistance was observed below this threshold. In nymphs, no threshold was found; *Borrelia* presence was associated with increased desiccation resistance. Interestingly, the *Borrelia*-associated effects were most profound for *B. afzelii* compared to other *Borrelia* genospecies. A study performed in 2008 using field-collected *I. ricinus* nymphs also shows an increased survival in *Borrelia* infected nymphs under desiccating conditions (Gassner, 2010).

A possible mechanism explaining increased desiccation resistance may be related to the tick's physiology of fat metabolism. Ticks store a relatively large quantity of brown fat, a high density and high energy form of fat storage in arthropods, during their blood meal, which is used as primary energy source during the in-between blood meal stage. The only way for *Ixodes* ticks to restore their water content is by active absorption of water vapour from the air. This process requires energy, which the tick can produce from the stored brown fat. An additional advantage of metabolising brown fat could be the generation of water (Chapman 1982), but no evidence exists for this in ticks. In both studies of Hermann and Gassner, fat content is indicated to play a key role in the increased desiccation resistance in infected ticks (Gassner 2010, Hermann and Gern 2010). Noteworthy, since the fat content is the tick's primary energy resource in between blood meals, increased activity of ticks may also be related to *Borrelia*-mediated changes in the tick fat metabolism.

This hypotheses is supported by observations on blood meal size and development in *Borrelia*-infected as well as uninfected *I. ricinus* feeding on field collected wood mice (*Apodemus sylvaticus*) (Gassner 2010). Here, blood meal size of larvae as well as post-moulting weight of infected nymphs was larger in *Borrelia*-infected ticks. These results would benefit from further evidence, since variation in the size of field collected larvae, which was not measured, may have had some effect. Moreover, feeding laboratory reared larvae on the same mice did not yield significant differences in engorgement size and post-ecdysis nymphal size.

In summary, *Borrelia* infections in ticks are associated with increased walking velocity, prolonged walking activity, increased desiccation resistance, and increased energy storage. The combined

effects of these observations will have a profound effect on tick survival and host finding. The potential contribution of this effect on the basic reproduction number of *Borrelia* is simulated below using a modelling approach.

Assessing the impact: a theoretical model

The effects described in the previous sections – the increased activity and survival of nymphs and adults will undoubtedly have an impact on the transmission of Lyme borreliosis. Due to the complexity of the system, it is hard to quantify the contribution of the described effects to the rate of *Borrelia* transmission in the field. However, a first assessment of the impact can be done by gauging the hypothetical effect on the basic reproduction number.

The basic reproduction number, or R_0, is defined as the average number of secondary cases caused by one infectious individual placed in a population consisting entirely of susceptibles (Diekmann *et al.* 1990). The value of R_0 is a measure of the likely success of invasion into a naïve population. If it is higher than 1, an outbreak of the disease is possible; if it is smaller than 1, the disease will die out. Also, in case of an outbreak, R_0 will determine the initial exponential increase in the number of inflected, and hence it is a measure for the (initial) transmission success. A method to derive the basic reproduction number for a tick-borne disease like Lyme borreliosis – using a next-generation matrix approach – has been described in Hartemink *et al.* (2008). Several of the parameters presented in this matrix model refer to processes that might be affected by the above-described fitness effects. The increased activity in infected ticks would result in a higher probability of finding a new host, since infected ticks can quest more actively and they are more likely to live long enough to find new hosts than uninfected ticks. In the matrix model, the host finding probability is not modeled explicitly; it is incorporated in the parameter describing the survival between life stages. This means that we can assess the effect of higher survival rate and increased activity in infected nymphs (and the resulting higher host finding rates) by using a higher value for the survival probability from feeding larva to feeding nymph (S_N). Similarly, by applying a higher value for S_A (the survival probability from feeding nymph to feeding female adult), we can gauge the effect of increased survival and activity in female adults.

Hence, we assess the hypothetical impact of the fitness effects by adjusting the estimates for S_N, and S_A and comparing the resulting R_0 values to the R_0 value based on the default parameter set. The parameters concerned are the survival probability from feeding larva to feeding nymph (S_N) and the survival probability from feeding nymph to feeding female adult (S_A). Based on the results described in this chapter, we used a conservative 50% increase for both parameters. For the other parameters, the values were the same as used in the paper by Hartemink *et al.* (2008). The sole exception is r_A, the per-egg probability of transovarial transmission; for this parameter we considered the original value (0.1) to be too high and we used 0.01 instead (a 1% probability per egg, in line with current insights (Rauter and Hartung 2005)). Since the fraction of blood meals taken on competent hosts can vary substantially, R_0 is shown as a function of this fraction, rather than as a single number.

In Figure 3 the different curves for R_0 are plotted: the bottom line reflects the R_0 values based on the default set of parameter values, whereas the other lines represents the curves for situation with a 50% increase in S_N, S_A or both.

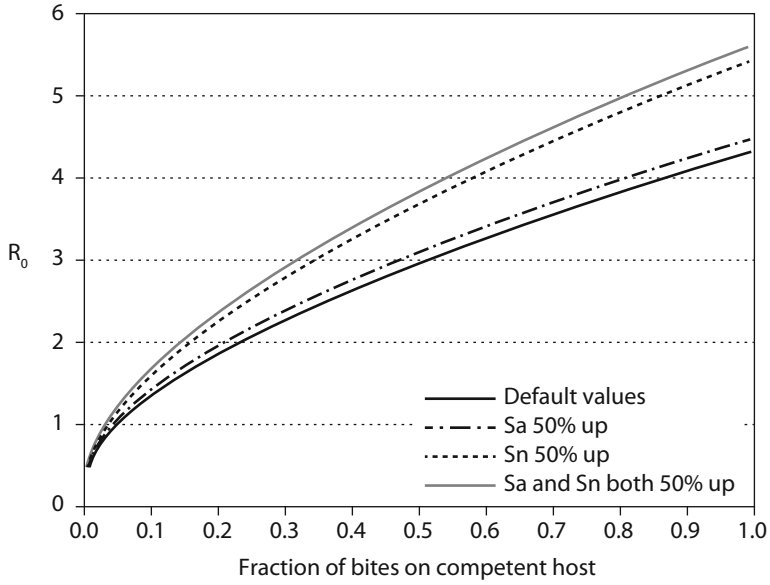

Figure 3. The effect of increased parameter values for S_N and S_A on the predicted value of the basic reproduction number R_0, based on a simple R_0 model. A 50% increase in the survival of nymphs (S_N) would have a considerable effect on the value of R_0, whereas a similar increase in the survival of adults (S_A) has less impact.

Interpretation of model

Even though the method applied here is based on rough estimates for parameter values and the modelled values for R_0 should not be interpreted as absolute values for any particular situation, our results clearly show that *Borrelia*-induced changes in survival of the tick would have an impact on disease transmission. Also, changes in nymph survival are likely to have more impact than changes in adult survival. This can be explained by looking at the biology of the transmission process.

Since transovarial transmission happens only occasionally, if at all, larvae are rarely infected (see for instance Zhioua *et al.* (1994) and Richter *et al.* (2012)). Larvae may pick up the infection during their first blood meal and moult into (infected) nymphs. These nymphs may in turn infect hosts, or other ticks, in the case of non-systemic transmission. The route from hosts to larvae that in their second blood meal infect hosts, is most probably the most important in the transmission cycle of Lyme borreliosis (see also Davis and Bent, 2011). For this route, the survival of feeding larva to feeding nymph is essential. It is therefore not surprising that an increase in this survival will have a large impact on total number of new cases per case, which here serves as a proxy for the transmission. The survival of feeding larva to feeding nymph is largely determined by two factors. The first factor is the survival of the nymph between blood meals, during which moisture stress is a key factor. The second factor is the success of unfed nymphs to successfully find a host and start their blood meal. *Borrelia*-associated effects are shown to affect both desiccation resistance

as well as tick activity. Both mechanisms potentially contribute strongly to the success of nymphs to successfully find a blood meal (S_N).

Parameter S_A, the survival of infected feeding nymphs to feeding female adult ticks, will affect the number of hosts that become infected through a bite of an infected female, but this number is comparatively low, because adult ticks usually do not feed on competent hosts. Therefore, the expected contribution of the adult ticks is lower and the increase in S_A has less effect than the increase in S_N.

Of course, a higher fitness of infected ticks may also affect other parameters in the model, such as the number of infected attached nymphs and adult ticks per host. However, since these effects are even more difficult to quantify, we choose to show only the most direct and straightforward effects, and indeed, these effects already indicate that the impact of the fitness effects on R_0 can be quite substantial.

Concluding remarks

Evidently, several *Borrelia*-associated modifications can be observed in ticks. We mentioned induced production of a protein that protects *Borrelia* against the host immune system, increased activity and increased desiccation resistance. The consequences of increased desiccation resistance may be that local dryer (micro)climate conditions in tick-infested habitats can select for higher densities of *Borrelia* infected ticks, resulting in local hot spots of *Borrelia* transmission.

We showed that in theory the R_0 of *Borrelia* can increase substantially if the *Borrelia*-associated modifications contribute to a better survival of feeding larvae to feeding nymphs. Such increased survival can benefit both the tick as *Borrelia*, and may hence be regarded as mutualistic interaction. In future studies epidemiologists should be aware of the differences in biology between infected and uninfected ticks to prevent an underestimation of Lyme borreliosis risk.

The questions that remain for future work are whether the observed *Borrelia*-associated changes affect tick-host contact and consequently the transmission rate of *Borrelia*. Increased understanding of the mechanisms in tick-*Borrelia*-host interactions may also contribute to the discovery of anti-tick vaccines (Schuijt *et al.* 2011). Eventually, preventing tick bites is more effective than finding a vaccine or cure against each of the many pathogens that can be transmitted by ticks and should therefore be among the priorities of strategies for the prevention of Lyme disease.

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8. *Wolbachia* in *Aedes* mosquitoes: towards biological control of vector-borne diseases

Luciano A. Moreira

Abstract

Dengue causes an enormous impact on global public health with 50 million cases every year. Vector control is predominantly focused on the application of insecticides against adult mosquitoes. However, current methods of vector control are often not sustainable for long periods because of the emergence of insecticide resistance. In this context, the discovery and application of alternative methods are extremely important. A new approach for biological control of diseases transmitted by mosquitoes has recently been proposed that uses an endosymbiotic bacterium (*Wolbachia pipientis*) in order to interfere with the transmission of pathogens. The advantage of using this bacterium is that infected females have a reproductive advantage due to a cytoplasmic incompatibility (CI), which leads to an increase in numbers of infected individuals in the wild. When *Aedes aegypti* mosquitoes (that naturally lack this bacterium) were transinfected with *Wolbachia* it was discovered that the presence of bacteria inhibits the replication of pathogens such as dengue and chikungunya viruses as well as filarial and avian malaria parasites. More recently *Ae. aegypti* mosquitoes harbouring a *Drosophila-Wolbachia* strain have been released in Australia and were able to quickly spread in the wild. In this chapter we review the possibility of applying this endosymbiotic bacteria as potential biological control of human diseases transmitted by mosquito vectors.

Keywords: *Aedes*, biological control, dengue, *Wolbachia*

Introduction

Vector-borne diseases such as malaria, leishmaniasis and dengue heavily impact on human mortality and morbidity throughout the world. With the increase of human movement (Adams and Kapan 2009) and the effects of global warming (Barclay 2008, Pachauri and Reisinger 2007) the expansion and resurgence of pathogens (Gould and Solomon 2008) such as dengue (DENV) and chikungunya (CHIKV) are becoming an increasing threat (Ng and Ojcius 2009, Staples *et al.* 2009). Dengue fever is being recently named as the most important disease affecting communities in tropical and sub-tropical regions around the world with 50 million cases annually and causing thousands of deaths (WHO 2009). A new approach to dengue control was proposed that targets mosquito longevity rather than their abundance, by introducing a strain of the bacterium *Wolbachia pipientis* (Hertig and Wolbach 1924), which causes a reduction in survival of *Aedes aegypti* L. mosquitoes (Brownstein *et al.* 2003, Cook *et al.* 2008, Rasgon *et al.* 2003, Sinkins and O'Neill 2000). Since the extrinsic incubation period of viruses and parasites within the mosquito vector is long (about 15 days) compared with the longevity of the insect (about 30 days in the field), the *Wolbachia* infection that may invade the mosquito population and causes a reduction in longevity of *Ae. aegypti*, is expected to reduce the transmission of pathogens without eliminating the mosquito population (Brownstein *et al.* 2003, Rasgon *et al.* 2003, Sinkins and O'Neill 2000). The *Wolbachia* wMelPop-CLA strain originating from *Drosophila melanogaster* Meigen was successcully introduced into *Ae. aegypti* (McMeniman *et al.* 2009). Mosquitoes containing this strain of *Wolbachia* died significantly sooner compared to their counterparts that lacked bacteria. Besides the effect on the insect longevity, and with great surprise was the discovery that the

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presence of bacteria in mosquitoes increases the resistance towards pathogens (Bian *et al.* 2010, Kambris *et al.* 2009, Moreira *et al.* 2009, Walker *et al.* 2011) as also shown in *Drosophila* flies for fly-specific viruses (Hedges *et al.* 2008, Teixeira *et al.* 2008). In this chapter we discuss the potential use of *Wolbachia* in biological control of diseases transmitted by mosquito vectors.

Phenotypic effects of *Wolbachia* in mosquitoes

Wolbachia are Gram-negative, obligatory intracellular bacteria, which manipulate host reproduction to ensure vertical transmission (from mother to offspring) (Sinkins *et al.* 1997). In the last decades *Wolbachia* was widely found infecting different species of invertebrates, with reports on arthropods such as insects (Jeyaprakash and Hoy 2000, Stouthamer *et al.* 1999, Werren *et al.* 1995), arachnids (Breeuwer and Jacobs 1996), crustaceans (Cordaux *et al.* 2001, Gotoh *et al.* 2003) and isopods, although they are also found in nematodes (Bandi *et al.* 1998, 2001). The first report of *Wolbachia* was in the reproductive tissues of *Culex pipiens* L. (Hertig and Wolbach 1924), being named *Wolbachia pipientis* (Hertig 1936). Recent statistical analysis confirmed the wide distribution of this bacterium among invertebrates, with an estimate of positivity in up to 65% of all insect species (Hilgenboecker *et al.* 2008), confirming previous findings of infection of 20 to 70% of insect species (Jeyaprakash and Hoy 2000).

In order to guarantee vertical transmission, the bacteria manipulate their hosts in various ways such as feminisation, male killing, parthenogenesis as well as through the mechanism of cytoplasmic incompatibility (Figure 1). As the latter is the most common phenomenon in mosquitoes this effect will be briefly explained below.

Cytoplasmic incompatibility

Cytoplasmic incompatibility (CI) is the most common effect caused by *Wolbachia* on arthropod reproduction. The phenotype results in the production of aberrant progeny originating from strains that harbour insects and various cytoplasmic factors that will affect the proper assembly of chromosomes in sperm soon after fertilisation. Typically, the paternal chromosomes are eliminated, leading to the formation of haploid embryos. In eggs of incompatible crosses, only the female pronucleus forms individual chromosomes and proceeds to the first division. The paternal

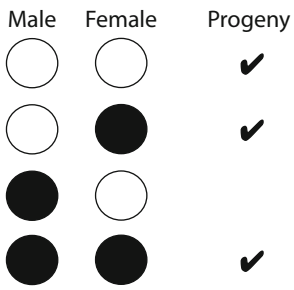

Figure 1. Unilateral mechanism of cytoplasmic incompatibility (CI). Crosses between infected males with uninfected females produce no viable offspring due to CI. Crosses involving Wolbachia*-infected females (in black) have higher chance of producing viable offspring, increasing the number of infected individuals in the population.*

pronucleus, which appears as a mass of chromatin tends to fragment during the first mitotic division. The effect of CI is usually unidirectional: incompatible crossing occurs between infected males and uninfected females, whereas reciprocal crosses between males free of bacteria and infected females produce normal offspring (Figure 1). However, there are reports of bidirectional incompatibility when species of insects (usually mosquitoes and *Drosophila*) hosts more than one strain of bacteria (Stouthamer *et al.* 1999, Yen and Barr 1971).

Wolbachia in *Aedes* mosquitoes

Early studies involved observations of *Rickettsia*-like organisms within mosquito ovaries and eggs. Following the work of Hertig in *Culex* (Hertig 1936), the first report of *Wolbachia* in mosquitoes came with the work by Yen (1975) on members of the *Aedes scutellaris* group (such as *Aedes cooki* Belkin, *Aedes polynesiensis* Marks, *Aedes albopictus* Skuse, *Aedes riversi* Bohart & Ingram). The ultrastructure of *Wolbachia* was then thoroughly studied in this same group by Wright and colleagues, who identified round shape structures within mosquito ovaries. They claimed that the nurse cells of the scutellaris group (unlike those in *Cx. pipiens*) are rarely infected with *Wolbachia* (Wright and Barr 1980).

The Asian tiger mosquito *Ae. albopictus* is native to Asia and the South Pacific and is an important vector of dengue and chikungunya viruses in some places of Southeast Asia (Kumari *et al.* 2011, Ratsitorahina *et al.* 2008, Rudnick and Chan 1965, Tsetsarkin *et al.* 2011). *Wolbachia* infections were first found in the ovaries of this mosquito (Dobson *et al.* 2001, 2004, Sinkins *et al.* 1995a, Wright and Wang 1980). Today it is well known that nearly all populations of *Ae. albopictus* harbour two different *Wolbachia* strains named *w*AlbA (A group) and *w*AlbB (B group) (Kittayapong *et al.* 2000, Ruang-Areerate *et al.* 2003, Sinkins *et al.* 1995a). Studies on the cytoplasmic incompatibility between both strains, towards the potential use of this feature as a mean of population suppression, have been performed by several groups (Dobson *et al.* 2001, 2004, Sinkins *et al.* 1995a, Tortosa *et al.* 2010).

Interestingly *Wolbachia* has never been found in wild *Ae. aegypti* (Kittayapong *et al.* 2000) nor in any anopheline mosquitoes (vectors of human malaria parasites) (Kittayapong *et al.* 2000, Rasgon and Scott 2004b, Ricci *et al.* 2002). The reason for this is unknown.

Transinfection of *Wolbachia* into *Aedes aegypti*

The success of *Wolbachia* transinfection between different insect taxa is dependent on the ability of this bacterium to adapt to new intracellular environments (Braig *et al.* 1994, Xi *et al.* 2005a). An example of successful transinfection in species of same genus involved the wAlbB *Wolbachia* strain from *Ae. albopictus* which was successfully established into *Ae. aegypti* using cytoplasmic transfer of embryos (Xi *et al.* 2005b). *Wolbachia*-infected mosquitoes exhibit cytoplasmic incompatibility and cage experiments have demonstrated that infected mosquitoes were able to reach fixation within seven generations (Xi *et al.* 2005b). Also, both *Wolbachia* strains from *Ae. albopictus* (*w*AlbA and *w*AlbB) were injected into *Ae. aegypti* adults and infected strains were able to persist over several generations (Ruang-Areerate and Kittayapong 2006).

However, the transfer of *Wolbachia* strains from other groups of insects seems to follow a prerequisite of cellular adaptation to the mosquito, which can be regarded as critical for successful transinfection. Thus, in order to facilitate the transfer of *Wolbachia* from *D. melanogaster* cells to *Ae. aegypti*, the *w*MelPop strain was first transferred to a mosquito cell line to allow adaptation to that intracellular environment (McMeniman *et al.* 2008). After continuous serial passage in mosquito

cell cultures for more than three years, the mosquito cell line became adapted to the strain of *Wolbachia,* named *w*MelPop-CLA (CLA-cell line adapted), which was later stably introduced into *Ae. aegypti* by microinjection of embryos (McMeniman *et al.* 2009). Two positive-*Wolbachia* strains were generated after a trial period of selection in early generations and both strains remained highly infected since then. Laboratory experiments using *Ae. aegypti* infected with wMelPop-CLA showed that the bacteria decreased adult longevity with approximately 50% (McMeniman *et al.* 2009, Yeap *et al.* 2011). This reduction in life expectancy of female *Ae. aegypti* may result in a significant decrease in the transmission of dengue virus by mosquitoes, if this ability to shorten the life in the laboratory ought to be reproduced under field conditions.

It is well known that the mosquito age is a critical factor for the transmission of pathogens (Dye 1992) as viruses and parasites go through an extrinsic incubation period (EIP) within the mosquito. The EIP is the time from ingestion of the pathogen until it is transmitted to the next vertebrate host and is a key component to calculate the vectorial capacity of diseases (Meyer 1989).

Influence of *Wolbachia* towards host-pathogen interactions

The interaction of *Wolbachia* with their hosts has the possibility of directly affecting their fitness (positively or negatively) or being regarded as silent or neutral. Therefore, the relation between the bacterium and the host can be regarded as mutualistic or parasitic (Brownlie *et al.* 2009, Werren *et al.* 2008).

In *Armadillidium vulgare* Latreille it has been shown that a particular strain of *Wolbachia* (*w*VulC) lowers haemocyte densities, increases septicaemia in their haemolymph and reduces their lifespan compared to individuals harbouring another bacterium strain or aposymbiotic (*Wolbachia*-free) ones (Braquart-Varnier *et al.* 2008). This phenomenon directly points to the fact that this microorganism can affect its host immunity. In another study, *Drosophila* infected with *Wolbachia* exhibited lower levels of encapsulation of parasitic wasp eggs than cured ones (Fytrou *et al.* 2006).

Recent studies in *Drosophila* have shown that infection with *Wolbachia* can protect flies from infection with RNA viruses (Hedges *et al.* 2008, Teixeira *et al.* 2008). The *Wolbachia* wMelPop and wMelCS strains that infect *D. melanogaster* induced reduction in mortality when the flies were infected with various pathogenic viruses including the *Drosophila* C virus, Flock House virus (FHV) and locusts paralysis virus (Cricket paralysis virus).

As the strain of *Wolbachia* wMelPop promoted protection against RNA viruses in *Drosophila*, it was important to test the effect of wMelPop-CLA on the vectorial competence of *Ae. aegypti* transinfected lines (McMeniman *et al.* 2009). For that, mosquitoes infected and uninfected with *Wolbachia* were exposed to dengue and Chikungunya viruses. The same mosquitoes were also tested with the avian malaria parasite, *Plasmodium gallinaceum*. The results showed that the presence of *Wolbachia* caused a drastic reduction in the presence or the development of these three unrelated pathogens, which opens new possibilities for controlling mosquito-borne diseases (Moreira *et al.* 2009). More recently it was shown that the bacterium (wMelPop-CLA) also provides protection against nematodes that cause lymphatic filariasis (Kambris *et al.* 2009) and blocked *Plasmodium falciparum* in somatically infected *Anopheles gambiae* Giles mosquitoes (Hughes *et al.* 2011), suggesting that some strains of *Wolbachia* may inhibit a wide range of human pathogens.

As viral interference is not ubiquitous among the strains of *Wolbachia* (Moreira *et al.* 2009, Osborne *et al.* 2009) the mechanisms behind the ability of bacteria to gain resistance against pathogens

are unknown. Although some effector genes (such as defensin, cecropin) were up-regulated in *Ae. aegypti* mosquitoes infected with *w*MelPop-CLA, key components of signalling pathways (Toll, IMD and Jak-STAT) do not appear to be transcriptionally modulated by *Wolbachia* (Kambris *et al.* 2010, Moreira *et al.* 2009). Previous studies have also revealed that some genes of the IMD and Jak-STAT pathways, involved in the control of infection by RNA viruses in insects (Huszar and Imler 2008), are differentially regulated in *Ae. aegypti* infected with dengue (Xi *et al.* 2008).

The ability of the CLA-wMelPop strain to provide protection against dengue virus may also be dependent on competition for essential components of host cells, as observed in infection with DENV-2 in cells infected with mosquito-CLA wMelPop (Moreira *et al.* 2009). Moreover, in *D. melanogaster* there is evidence that strains of *Wolbachia* get much of their energy through the metabolism of amino acids (Wu *et al.* 2004), including threonine – an amino acid required in the activation of expression of vitellogenin (Vg) in *Ae. aegypti* (Attardo *et al.* 2006). Recently McMeniman and colleagues hypothesized the existence of a competition between wMelPop *Wolbachia* and *Ae. aegypti* to obtain threonine required for expression of Vg and subsequently inhibition of egg development (McMeniman *et al.* 2011). Alternatively, it is known that insects also need to get cholesterol and other fatty acids in the diet (Blitzer *et al.* 2005) and, as the *Wolbachia* and other bacteria do not synthesise cholesterol, they might need to get it from the insect host (Lin and Rikihisa 2003, Wu *et al.* 2004). Cholesterol is known to be a key fatty acid necessary for successful replication of flaviviruses and that must be obtained from the host cell (Lu *et al.* 1999, Mackenzie *et al.* 2007). Likewise *Plasmodium* also depend on the mosquito lipids (Atella *et al.* 2009), suggesting that cholesterol may be a critical nutrient required by both the pathogen and the *Wolbachia* within the mosquito.

The distribution of *Wolbachia* in different tissues of the mosquito, as well as the density of the same insect host cells may be an important determinant in the ability of bacteria to interfere with the pathogens. *Wolbachia* strains that provide protection in *Drosophila simulans* Sturtevant, are closely related to wMelPop from *D. melanogaster* and are also found in high densities in flies (Osborne *et al.* 2009). But recently, the non-virulent strain *w*Mel (also from *Drosophila*), although present in much lower densities than *w*MelPop, promotes significant protection against DENV-2 in transinfected *Ae. aegypti* mosquitoes, resulting in a total blockage of dengue transmission under experimental conditions (Walker *et al.* 2011). Perhaps, a combination of bacterial tissue tropism, host immunity upregulation and competition for host cell resources is what is needed for pathogen blockage.

Contrarily to observations in *Drosophila*, the strains of *Wolbachia* (Hedges *et al.* 2008, Teixeira *et al.* 2008) which naturally reside in mosquitoes, have very limited ability to protect against viruses. *Ae. albopictus* infected with the non-virulent strains of *Wolbachia* (wAlbA and wAlbB) (Sinkins *et al.* 1995b) are still vectors of dengue virus (Kyle and Harris 2008). Likewise, *Armigeres subalbatus* Coquillett mosquitoes infected with another strain of *Wolbachia* showed no evidence of interference with the Japanese encephalitis virus (Tsai *et al.* 2006). Recently, the strain of *Wolbachia w*Pip native of *Culex quinquefasciatus* Say, was shown to have some protective effect against West Nile virus (Glaser and Meola 2010). However, this effect was much less pronounced when compared with the effects on dengue virus in transinfected *Ae. aegypti* (Moreira *et al.* 2009, Walker *et al.* 2011).

Practical application

The practical approach of using *Wolbachia* to control the spread of mosquito-borne diseases has been around for a long time (Curtis and Sinkins 1998, Rasgon and Scott 2004a, Sinkins *et al.* 1997). The mechanism of CI has been experimentally used in the wild in order to eradicate a population of *Cx. quinquefasciatus* (formerly: *Culex pipiens fatigans*) in Burma. Although adult numbers decreased to very low levels, the authors were not able to completely eliminate the local vector population (Laven 1967). More than a decade ago it has been proposed to use *Wolbachia* as a tool for spreading gene(s) of interest in field populations. The initial idea was to transform this bacterium by placing, for example, a refractoriness gene and, with the action of cytoplasmic incompatibility (CI) the bacteria would invade the wild population carrying the gene of interest (Curtis and Sinkins 1998). However since then, several laboratories have been trying to transform the bacteria with no success and the inability of this microorganism to live in a cell free medium appears to be the main impairment.

The CI mechanism has been proposed as an alternative to the Sterile Insect Technique (SIT) where males harbouring a different *Wolbachia* strain from the one present in one particular area could be released and suppress local mosquito population by effectively sterilizing their female counterparts (Calvitti *et al.* 2010). *Ae. polynesiensis* is the main vector of human filariasis in the South Pacific. The use of incompatible *Wolbachia*-infected mosquito strains is been sought as infected males can be released and then decrease natural mosquito populations through the CI mechanism (Chambers *et al.* 2011).

The discovery that certain strains of *Wolbachia* may interact with pathogens by blocking their development in mosquitoes shifted the focus for the use of this bacterium and the previous life-shortening approach has been put on hold in favour of other, more effective, strategies. More recently, the use of *Wolbachia*-transinfected mosquito strains shows the potential of causing significant effects in disease control programmes around the world.

After a long and broad risk analysis step performed by two Australian research and regulatory agencies (CSIRO and APVMA) (Murphy *et al.* 2010) the release of *Wolbachia*-infected mosquitoes in the wild has been approved (DeBarro *et al.* 2011). In the beginning of January 2011 two localities that previously had undergone through a vast programme of community engagement, received the first *Wolbachia*-positive *Ae. aegypti* mosquitoes in nature (http://www.eliminatedengue.org). This process was followed by 9-10 weeks of adult mosquitoes releases and even with the occurrence of a cyclone they were able to invade natural populations, reaching up to 100% after the introduction has been halted (Hoffmann *et al.* 2011) (Figure 2). Soon the same approach will be used in other countries as Vietnam, Thailand, Indonesia and Brazil. If the *Wolbachia*-positive mosquitoes released in the field have the same ability of blocking the dengue virus the way it has been shown in laboratory conditions it will bring a big impact on disease control in the world. Further studies using the available *Wolbachia-Aedes* strains towards other dengue serotypes and other arboviroses such as yellow fever are most welcomed to expand the potential use of this strategy towards other diseases (Van den Hurk *et al.* 2012).

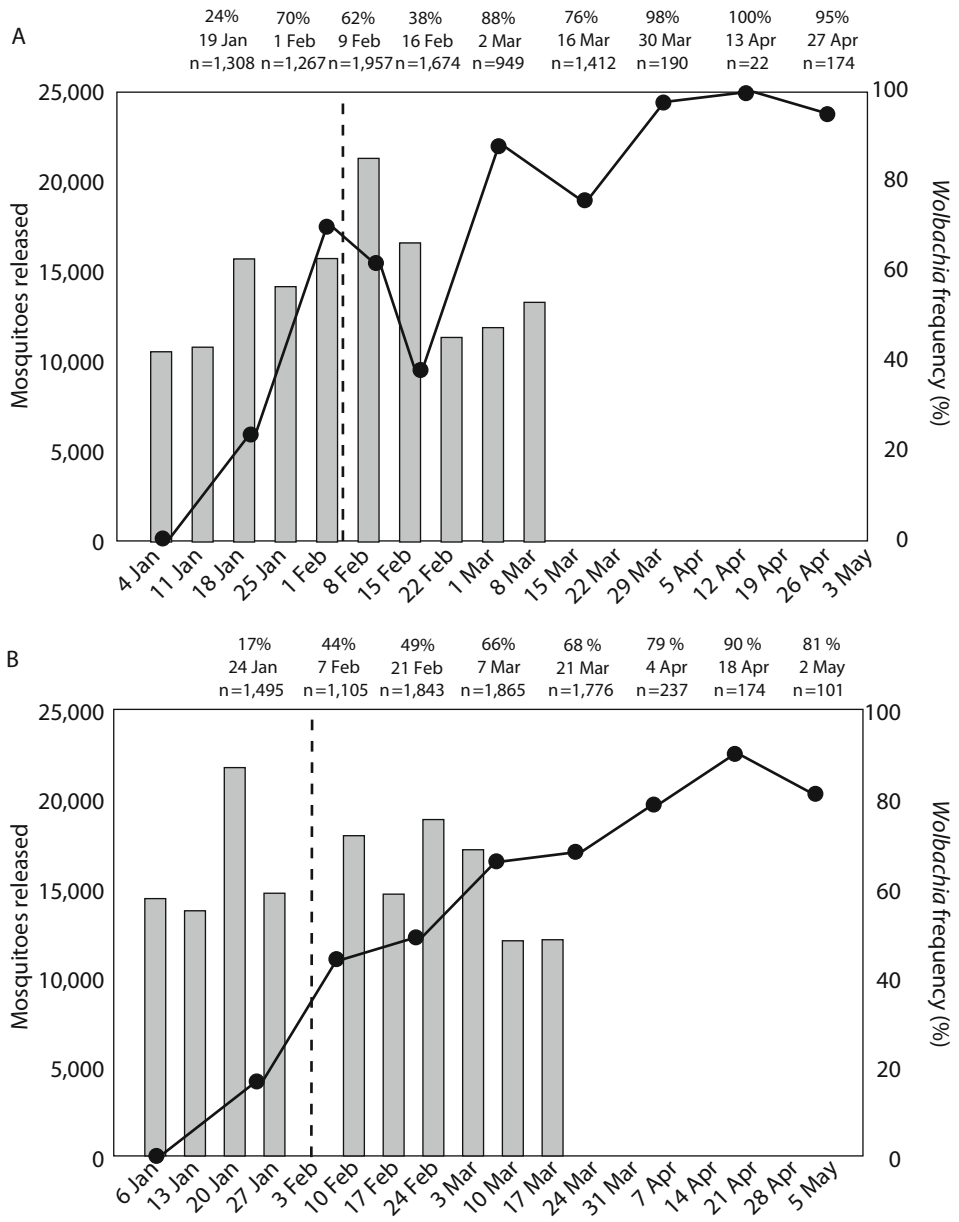

Figure 2. Number of mosquitoes released, timing of releases, and changes in infection frequencies over time. Data based on monitoring with ovitraps at (A) Yorkeys Knob and (B) Gordonvale. Ten releases were carried out at each site. Lower numbers were collected late in the season because of a reduction in trapping intensity and the advent of the dry season. Tropical Cyclone Yasi landed on 3 February (dotted line) and disrupted Wolbachia *monitoring collections at Yorkeys Knob. A planned release at Gordonvale on 3 February was cancelled (Hoffmann* et al. *2011).*

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9. Behaviour of sandflies infected with *Leishmania*

Paul D. Ready and Matthew E. Rogers

Abstract

Leishmaniasis is a 'neglected tropical disease', and there have been some significant recent advances in research on how *Leishmania* species, the causative agents of the disease, affect the behaviour of phlebotomine sandflies, their insect vectors. This chapter briefly describes the eco-epidemiology of some natural transmission cycles, focusing on the ones that are susceptible to laboratory experimentation. Research on these transmission cycles has often been prompted by their public health importance, although some vector-parasite associations have become models because of the ease of laboratory experimentation. The principal models of experimental infection are explained in some detail, to enable us to review the current knowledge of changes in sandfly behavioural traits that have been specifically related to *Leishmania* infections and might affect leishmaniasis transmission. Finally, we discuss how this knowledge could lead to new ways of controlling sandflies and leishmaniasis.

Keywords: *Leishmania* manipulation, parasite-vector interactions, phlebotomine behaviour, phlebotomine sandflies.

Introduction: eco-epidemiology of natural sandfly-*Leishmania* associations

Leishmaniasis is a 'neglected tropical disease', causing a high burden of disease in affected areas (WHO 2012). The parasites are vectored by phlebotomine sandflies. There have been some significant recent advances in research on how *Leishmania* species, the causative agents of the disease, affect the behaviour of phlebotomine sandflies, their insect vectors. The aims of this chapter are firstly to review the current knowledge of changes in sandfly behavioural traits that have been specifically related to *Leishmania* infections and might affect leishmaniasis transmission, and secondly to discuss how this knowledge could lead to new ways of controlling sandflies and leishmaniasis.

The main models of experimental infection will be explained in the Section 'Experimental models of *Leishmania*-sandfly interactions', but first we set out briefly the eco-epidemiology of their natural transmission cycles (Table 1). Research on these transmission cycles has often been prompted by their public health importance, although some vector-parasite associations have become models because of the ease of laboratory experimentation.

Old World zoonotic cutaneous leishmaniasis and anthroponotic cutaneous leishmaniasis

Old World (OW) zoonotic cutaneous leishmaniasis (ZCL) is mostly caused by *Leishmania (Leishmania) major* Yakimoff and Schokhor, which is specifically transmitted by *Phlebotomus (Phlebotomus) papatasi* Scopoli in North Africa and southwest Asia, *Phlebotomus (Phlebotomus) duboscqi* Neveu-Lemaire in sub-Saharan Africa and probably *Phlebotomus (Phlebotomus) salehi* Mesghali in southeast Iran, Pakistan and northwest India (Desjeux 2001, Killick-Kendrick 1990, Ready 2013). There are often tens of thousands of new human cases each year (WHO 2012), and most transmission occurs in arid rural environments where people live close to the gerbil and other rodent reservoir hosts (Parvizi and Ready 2008). In addition to the public health importance of ZCL, the transmission of *Le. major* by *P. papatasi* is an important laboratory model because

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*Table 1. Natural sandfly-*Leishmania *associations and laboratory models of experimental transmission. Of all these species, only those in subgenus* Leishmania (Viannia) *usually develop in the sandfly hindgut before anterior migration.*

Leishmaniasis	Region (bioclimate)	Parasite	Principal vector species or subgenus	Main references to laboratory models
Anthroponotic cutaneous leishmaniasis (ACL)	Africa, Asia (semi-arid)	*Leishmania (Leishmania) tropica*	*Phlebotomus (Paraphlebotomus) sergenti* *Phlebotomus (Adlerius) arabicus*	Killick-Kendrick (1990) Svobodová *et al.* (2006)
Zoonotic cutaneous leishmaniasis (ZCL)	Africa, Asia (arid, semi-arid)	*Leishmania (Le.) major*	*Phlebotomus (Phlebotomus) papatasi* *Phlebotomus (Phlebotomus) duboscqi*	Dobson *et al.* (2010) Sadlova and Volf (2009)
	Neotropics (equatorial, sub-humid)	*Leishmania (Viannia) braziliensis* and related species	*Lutzomyia (Helcocyrtomyia)* species *Lutzomyia (Nyssomyia)* species *Lutzomyia (Psychodopygus)* species *Lutzomyia (Trichophoromyia)* species *Lutzomyia* species group Migonei *Lutzomyia* species group Verrucarum	Killick-Kendrick (1990) Walters *et al.* (1989)
		Leishmania (Le.) mexicana and related species	*Lutzomyia (Nyssomyia) flaviscutellata* complex	Killick-Kendrick (1990)
			Lutzomyia (Lutzomyia) longipalpis (Experimental vector)	Bates (2007) Rogers *et al.* (2002) Rogers and Bates (2007)
Anthroponotic visceral leishmaniasis (AVL)	Indian subcontinent, north-east Africa (sub-humid)	*Leishmania (Le.) donovani*	*Phlebotomus (Euphlebotomus) argentipes* *Phlebotomus (Larroussius) orientalis*	Killick-Kendrick (1990) Molyneux and Killick-Kendrick (1987)
Zoonotic visceral leishmaniasis (ZVL)	Mediterranean, Asia, neotropics (semi-arid, sub-humid, equatorial)	*Leishmania (Le.) infantum* (Member of *Le. donovani* complex)	*Phlebotomus (Larroussius)* species *Lutzomyia longipalpis* *Lutzomyia evansi* species group Verrucarum	Killick-Kendrick (1990) Molyneux and Killick-Kendrick (1987) Rogers *et al.* (2010)

of the ease of culturing all the parasite developmental stages, colonizing this sand fly species, performing experimental transmissions to inbred mice (Kamhawi 2006) and even demonstrating genetic exchange between parasites within the vector (Akopyants *et al.* 2009).

In contrast, much less experimental research has been performed on the transmission of the other widespread causative agent of OW cutaneous leishmaniasis, *Leishmania (Leishmania) tropica* Wright (Killick-Kendrick 1990, Svobodová *et al.* 2006), and this can be explained by the greater difficulties of culturing these parasites and vectors as well as a lower public health profile. This parasite occurs in many of the same regions as *Le. major*, but it is usually associated with urban areas where it causes OW anthroponotic cutaneous leishmaniasis (ACL) and is transmitted by *Phlebotomus (Paraphlebotomus) sergenti* Parrot and related vectors, although it can cause ZCL and *Phlebotomus (Adlerius) arabicus* Theodor is an incriminated vector (Svobodová *et al.* 2006, Volf and Myskova 2007).

New World zoonotic cutaneous leishmaniasis

There are far more *Leishmania* species causing New World (NW) ZCL (Lainson *et al.* 1994). Those classified in the subgenus *Leishmania* are mostly in the *Leishmania mexicana* species complex, including *Le. mexicana* Biagi and *Leishmania amazonensis* Lainson and Shaw. These are easily cultured to produce developmental stages equivalent to those of *Le. major*, but unfortunately the natural vectors of the *Lutzomyia (Nyssomyia) flaviscutellata* Mangabeira complex are not so experimentally tractable (Killick-Kendrick 1990). Consequently, a permissive but unnatural vector, *Lutzomyia (Lutzomyia) longipalpis* (Lutz and Neiva), is often used for experimental transmissions (Rogers *et al.* 2002, 2004, 2008).

The vectors of the *Le. mexicana* species complex are often abundant in non-climax forests (Ready *et al.* 1983). In contrast, the more diverse *Lutzomyia* vectors of NW *Leishmania (Viannia)* species are usually more abundant in tropical forests and formerly forested regions (Killick-Kendrick 1990, Lainson *et al.* 1994, Ready 2013), where most NW ZCL is acquired (Desjeux 2001, WHO 2012). Unfortunately, neither the parasites, usually *Leishmania (Viannia) braziliensis* Vianna and related species, nor most of their vectors are suited to routine laboratory experimentation (Killick-Kendrick 1990).

Anthroponotic visceral leishmaniasis

Anthroponotic visceral leishmaniasis (AVL) is restricted to the Old World, and most transmission of the causative agent, *Leishmania (Leishmania) donovani* Laveran and Mesnil, is by *Phlebotomus (Euphlebotomus) argentipes* Annandale and Brunetti (Killick-Kendrick 1990) in peridomestic habitats in northeast India and nearby Bangladesh and Nepal (Bern *et al.* 2010).

Zoonotic visceral leishmaniasis

Unlike AVL, zoonotic visceral leishmaniasis (ZVL) occurs in both the Old World and the New World. This probably results from the causative agent, *Leishmania (Leishmania) infantum* Nicolle, being imported to the Americas in the reservoir host, the domestic dog (Quinnell and Courtenay 2009), and finding there a permissive vector, *L. longipalpis* (Killick-Kendrick 1990, Volf and Myskova 2007), that was pre-adapted to peridomestic habitats (Ready 2013). The OW vectors are usually *Phlebotomus (Larroussius)* species (Ready 2010).

Experimental models of *Leishmania*-sandfly interactions

As pool feeders, the females of *Phlebotomus* and *Lutzomyia* species obtain blood meals from mammals by using their mouthparts to break superficial capillaries and surrounding cutaneous tissues. Damage to the skin may release amastigotes of *Leishmania* that live within the phagolysosomes of macrophages and other phagocytes (Bates 2007, Kamhawi 2006). Shortly after taking a blood meal, the increase in pH and decrease in temperature in the midgut of the sandfly stimulate the parasite's transformation from the superficially aflagellated and rounded amastigote to the flagellated, more elongated and motile procyclic promastigote (Figure 1). This initiates a sequence of further morphological and molecular transformations, culminating in the occurrence of metacyclic promastogotes, the form infective to mammals, in the anterior thoracic midgut and foregut. This process has been confirmed by many observers of the development of *Le. major* in *P. papatasi* and a few *Leishmania* species in *L. longipalpis*, but the details for most *Leishmania*-sandfly combinations are either unknown or rely on limited reports (Table 1), as illustrated by the following examples.

Old World zoonotic cutaneous leishmaniasis models

Among the best studied interactions are those between *Le. major* and its natural vectors *P. papatasi* and the related *P. duboscqi* (Bates 2007, Kamhawi 2006). Two main waves of parasite loss occur in infected flies, corresponding to the digestion of the blood meal and its defecation (Pimenta *et al.* 1997, Rogers *et al.* 2008). The weakly motile procyclics divide in the blood meal within the sandfly's peritrophic membrane, a chitinous matrix secreted by the midgut. This matrix (Pimenta *et al.* 1997) and *Leishmania* secreted phosphoglycans (Kamhawi 2006) protect the procyclics from the sandfly's digestive proteases, including trypsins (Secundino *et al.* 2010). Transformation to the more motile nectomonad stage occurs on day 2-3, and coincides with disintegration of the peritrophic membrane. *Le. major* nectomonad promastigotes are known to congregate at the anterior end of the peritrophic membrane, suggesting that they are released by the action of their own chitinase (Shakarian and Dwyer 2000) and perhaps that of the sandfly (Ramalho-Ortigao *et al.* 2005, Sadlova and Volf 2009). It appears that nectomonads can only colonize the anterior thoracic midgut after the peritrophic membrane is completely broken down by sandfly chitinases (Sadlova and Volf 2009). Midgut peristalsis helps excrete the blood meal remains, and Vaidyanathan (2005) identified a secreted myoinhibitory neuropeptide of *Le. major* that arrested peristalsis. The non-replicating nectomonads have a predominant surface lipophosphoglycan (LPG) (Dobson *et al.* 2010), and this glycoconjugate protects them against agglutinating sandfly lectins (Kamhawi 2006) and permits specific attachment to the epithelium of midgut microvilli by binding to a galectin (Kamhawi *et al.* 2004), without which they can be expelled in the blood meal remains. The nectomonads migrate to the anterior thoracic midgut, where they transform by day 4 to leptomonad promastigotes (Kamhawi 2006). These replicate and by day 5-7 form large clusters around the stomodeal valve, at the junction of midgut and foregut. By day 6-9 some leptomonads have transformed to infective-stage metacyclic promastigotes, which were associated with a glycoconjugate-containing plug by Davies *et al.* (1990), one that might block the smooth ingestion of a subsequent bloodmeal and thereby facilitate transmission by regurgitation. Until the report of Killick-Kendrick and Rioux (2002), there were still supporters of an alternative hypothesis, namely that metacyclics and related forms had to reach the proboscis for transmission by inoculation (Bates 2007).

This model contains most of the interactions between *Leishmania* and the sandfly alimentary track, but only rarely has it been inferred that any interaction can alter sandfly behaviour in a way that

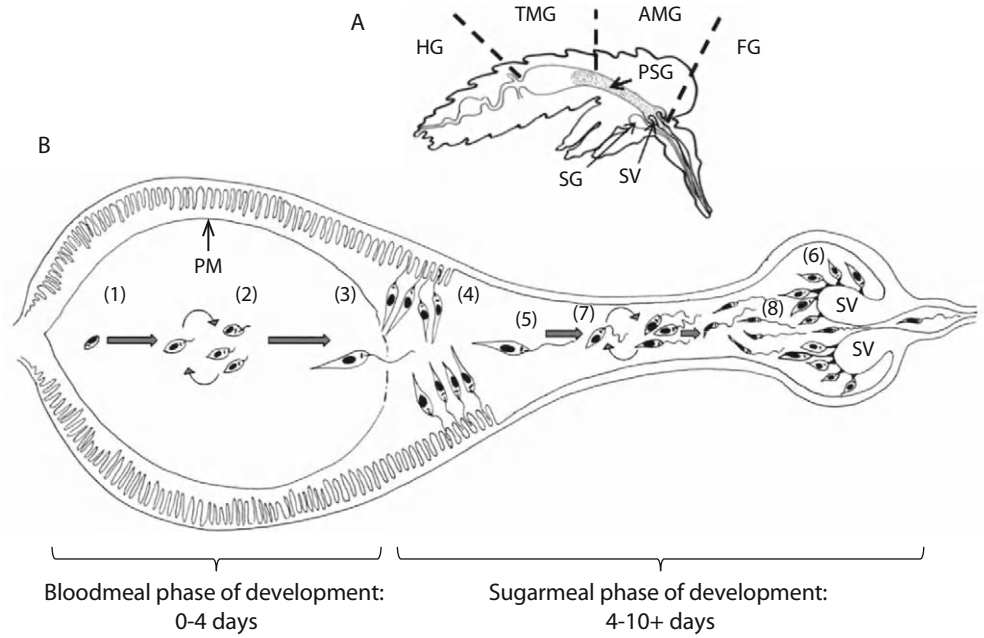

Figure 1. Typical development in a sandfly host of Leishmania (Leishmania) *species. (A) Anatomy of sandfly alimentary tract indicating position of promastigote secretory gel (PSG – stippled area). (B)* Leishmania *development in the sandfly midgut: (1) Amastigotes enter sandfly midgut when a sandfly bloodfeeds from an infected host. (2) Within 12-24 hours the amastigotes transform into a weakly motile, procyclic promastigote form which multiplies within the digesting bloodmeal. (3) At the end of digestion the procyclics transform into the non-divisional nectomonad forms which migrate through the peritophic matrix (PM) into the ectoperitophic space, before the bloodmeal is defecated. (4) Nectomonads attach to the microvilli of the midgut to resist expulsion by defecation then detach and resume their forward migration from the midgut (TMG) to the anterior thoracic midgut (AMG). (5) The majority of nectomonads transform into leptomonad promastigotes and resume multiplication in the anterior thoracic midgut. (6) It is suspected that a smaller proportion of nectomonads or leptomonads that first arrive at the stomodeal valve (SV) initiate a biological plug of parasites by attaching to the chitinous surface of the valve. To do this these haptomonad promastigotes modify their flagellum into a hemidesmosome-like attachment plaque. (7) Leptomonads are the main producers of filamentous proteophosphoglycan, which polymerises into a gel-like blockage in the anterior gut (the PSG) which allows parasites to access the foregut (FG) and promotes a regurgitative mode of transmission. (8) Finally, leptomonads differentiate into the mammal-infective metacyclic promastigotes which are deposited into skin when the sandfly takes another bloodmeal. HG, hindgut. SG, salivary gland. Not to scale.*

modifies transmission (see Section 'Manipulation of *Leishmania* of sandflies feeding on mammalian hosts'). Most interactions would seem to lead to adaptive selection of parasite strains and species capable of developing in their regional sandfly vectors. For example, the West African Seidman strain of *Le. major* has LPG permitting growth in the African *P. duboscqi* but not in the Mediterranean and Asiatic *P. papatasi* (Joshi *et al.* 2002), and some Asiatic strains of *Le. major* only develop in their regional populations of *P. papatasi* (Dobson *et al.* 2010, Kamhawi 2006). Experimental evidence is required to demonstrate manipulation of sandfly behaviour, for example to show that parasite

chitinase might shorten the time between sandfly blood meals by causing the early rupture of the anterior peritrophic membrane. The chitinase of *Le. major* does damage the stomodeal valve of *P. papatasi* (Schlein *et al.* 1992), but there is no proof that this causes a change in sandfly behaviour facilitating transmission, namely regurgitation, as it may be only a pathogenic by-product of infection (Bates 2007). Little is known about sandfly defence against *Leishmania* infections and any modulation of this by the parasite. In addition to sandfly lectins (Kamhawi 2006), a defensin of *P. duboscqi* has anti-*Le. major* activity (Boulanger *et al.* 2004), but the activity was not shown to be specific and so it need not demonstrate adaptive selection by this sandfly to the parasite. The best evidence for parasite modulation of sandfly defences comes from the species specificity of the myoinhibitory neuropeptide of *Le. major*. It arrested peristalsis in the sandfly hindgut, fully in *P. papatasi* but incompletely in *L. longipalpis*, and 100% inhibition was caused only by lysates of *Le. major*, not those of *Le. donovani* or *Le. braziliensis* (Vaidyanathan, 2005).

New World zoonotic cutaneous leishmaniasis models

As already mentioned (Section 'Introduction: eco-epidemiology of natural sandfly-*Leishmania* associations', Table 1), *Le. mexicana* is not naturally transmitted by *L. longipalpis*, even though they both occur in the neotropics, and so the interactions between them are not necessarily those of a co-evolving parasite and vector. However, they do provide a robust laboratory model, one that has not only demonstrated a pattern of infection similar to that of *Le. major* in *P. papatasi*, but also has revealed novel aspects of *Leishmania* subgenus-vector interactions. Shared characteristics (Bates 2007, Rogers *et al.* 2002) include the protection against proteases afforded by procyclic secreted phosphoglycans and the sandfly peritrophic membrane, the release of nectomonads from the latter by chitinases (Ramalho-Ortigao *et al.* 2005, Rogers *et al.* 2008, Sant'Anna *et al.* 2009), the defensive properties of sandfly lectins and *Leishmania* inhibitors of sandfly gut peristalsis (Kamhawi 2006), the sequence of parasite transformations and forward migration, and the blockage caused by a stomodeal plug that facilitates transmission by regurgitation (Rogers *et al.* 2002, 2004; Stierhof *et al*., 1999). However, key differences are: the protective role (in the closely related *Le. amazonensis*) of the major parasite surface glycoprotein, the metalloproteinase gp63 or leishmanolysin, in the early stage of infection in *L. longipalpis* but not *P. duboscqi* (Hajmová *et al.* 2004); nectomonad midgut binding not mediated by LPG (Rogers *et al.* 2004; Kamhawi 2006); and, the demonstration that chitinase enhances transmission to mice (Rogers *et al.* 2008).

Novel findings have flowed from establishing that the stomodeal blockage is caused by a promastigote secretory gel (PSG), which contains mostly filamentous proteophosphoglycan (fPPG) secreted by leptomonads as they multiply in the anterior midgut (Rogers *et al.* 2004). Finally, leptomonad promastigotes differentiate into the infective metacyclic promastigotes which are regurgitated with the PSG during blood-feeding. The fPPG component of PSG causes substantial exacerbation of disease in mice (Rogers *et al.* 2004, 2009). Moreover, the blockage of the stomodeal valve (Rogers and Bates 2007) and the damage caused it by chitinase (Rogers *et al.* 2008) have been shown to manipulate the behaviour of *L. longipalpis* such that transmission is enhanced (see Section 'Manipulation of *Leishamania* of sandflies feeding on mammalian hosts').

There are no laboratory models for routinely investigating how *Leishmania (Viannia)* species develop in their natural neotropical vectors, and so there have been no demonstrations of parasite manipulation of sandflies. However, the development of *Le. braziliensis*, *Leishmania (Viannia) panamensis* Lainson and Shaw and related parasites have been observed in the unnatural vector *L. longipalpis* (Bates 2007), suspected vectors such as *Lutzomyia migonei* França (Nieves and Pimenta 2000) and other *Lutzomyia* species (Walters *et al.* 1989). Characteristically, *Leishmania (Viannia)*

species establish infections by attaching to the pylorus and hindgut after escaping from the peritrophic membrane. Despite this different route, and the possibility of midgut attachment by metacyclic-like forms (Kamhawi 2006), the end result is the secretion of PSG and its association with metacyclics in the anterior thoracic midgut of the sandfly (Bates 2007).

Anthroponotic visceral leishmaniasis models

It has not been possible to investigate routinely the development of infections of *Le. donovani* in *P. argentipes*, because of the difficulty of establishing colonies of this sandfly species. However, 20th century transmission studies in India reported parasite forms and development patterns consistent with those of *Le. major* and *Le. mexicana* (Molyneux and Killick-Kendrick 1987). We could find no experimental demonstrations of *Leishmania* manipulating the behaviour of *P. argentipes*, even though it is susceptible to the infection of several *Leishmania* species, including *Le. major* and *Le. tropica* (Kamhawi 2006).

Zoonotic visceral leishmaniasis models

The parasite is believed to have an Old World origin, and so it is interesting that the development of promastigotes of *Le. infantum* has been studied in a wild population of one of its European vectors, *Phlebotomus ariasi* Tonnoir, in a rural zoonotic visceral leishmaniasis (ZVL) focus in southern France (Killick-Kendrick and Rioux 2002). Wild females were captured, offered blood meals on an infected dog, marked with a fluorescent powder unique for the day, released and then recaptured. Of 253 females recaptured, 124 contained promastigotes 7-29 days after feeding on the infected dog, and parasite development in the natural environment was similar to that of the *Leishmania* species mentioned above. The findings were reported to be consistent with transmission by regurgitation about 13 days post-infection, with routine transmission by inoculation without regurgitation being dismissed because parasites were found in the pharynx and proboscis only from days 19 and 22, respectively (Killick-Kendrick and Rioux 2002).

There have been many observations of the development of neotropical *Le. infantum chagasi* in its natural vector *L. longipalpis* (Molyneux and Killick-Kendrick 1987), including experimental transmission 7 days post-infection in the absence of proboscis infections (Lainson *et al.* 1977), a pattern consistent with transmission by regurgitation. A recent report demonstrated that PSG and metacyclics of *Le. infantum chagasi* are regurgitated by *L. longipalpis* and that the fPPG promoted the establishment of infections in mouse skin and the spleen (Rogers *et al.* 2010). This expands research on this parasite-vector combination – on metacyclogenesis, PSG production and biting persistence (Rogers and Bates 2007) – and the protocols in both papers provide a framework for a more natural experimental model.

Manipulation by *Leishmania* of sandflies feeding on mammalian hosts

Parasites exhibit many adaptations to ensure their transmission from one host to another. Manipulation is a particular type of adaptation, one where an infection elicits a specific behavioural response from the host of selective benefit to the parasite (Hurd 2003, Lefèvre *et al.* 2006). In this strict sense, there are only two reports (Rogers and Bates 2007, Rogers *et al.* 2008) that demonstrate manipulation of sandfly feeding behaviour by *Leishmania*. These two cases identify the manipulator molecules that enhance transmission, fPPG and chitinase respectively, and their effects on transmission, which has rarely been achieved for any investigation of parasite manipulation. The successful demonstration of sandfly manipulation by *Leishmania* was made

possible by using two of the models already described, namely the transmission of *Le. mexicana* and *Le. infantum* by *L. longipalpis*. There is clearly much scope for extending such investigations (see Section 'Evolution of *Leishmania* manipulation of sandfly behaviour') to other pairs of *Leishmania* and sandfly species, to different aspects of sandfly behaviour, and to other *Leishmania*-sandfly interactions. So far, the findings only relate to the two-way interactions within the sandfly and how these affect transmission by changing blood feeding persistence and duration, which are three-way interactions because they involve the mammalian host. Remaining to be investigated are the two-way *Leishmania*-mammal interaction and the three-way interaction when sandflies acquire an infection from a mammalian reservoir.

Rogers and Bates (2007) were the first to provide experimental support for the long-held assumption that *Leishmania* can manipulate the feeding behaviour of its sandfly vectors. They concluded that the parasite manipulates the timing of sandfly blood feeding to ensure that it coincides with peak numbers of infective metacyclics in and around the PSG plug that blocks the stomodeal valve and other parts of the anterior thoracic midgut. Parasite secretion of PSG was associated with differentiation of metacyclics, and the plug together with the metacyclics caused an increase in vector biting persistence on mice, as measured by re-feeding after interruption, and also promoted feeding on multiple hosts. The development rate of the parasites was experimentally accelerated, in order to demonstrate that *Leishmania* induced increased biting persistence only when metacyclics were present. The manipulation enhanced the infection of experimental hosts, demonstrating that transmission was optimised and providing a selective advantage to the parasite. This was the best evidence for concluding that the behavioural manipulation is adaptive (Rogers and Bates 2007), but other support (Hurd 2003) came from the complexity of the manipulation, the evidence of 'purposive design', and the independent origins of fPPG gel in other *Leishmania*-sand fly combinations. Rogers and Bates (2007) speculated that blockage by the PSG plug might promote the hunger state or alternatively increase the threshold blood volume required to inhibit blood-seeking behaviour.

In the second report on behavioural manipulation, Rogers *et al.* (2008) demonstrated that experimental over-expression of *Leishmania* chitinase gave rise to heavier stomodeal valve infections and longer feeding times, leading to faster evolving cutaneous lesions and larger final lesions in mice.

Evolution of *Leishmania* manipulation of sandfly behaviour

The question arises whether the sandfly has needed to respond to this behavioural manipulation by *Leishmania*. Has the manipulation introduced fitness costs for the sandfly? The answer is no, at least for the lifespan and fecundity of *L. longipalpis* in the laboratory (Rogers and Bates 2007). In natural environments, however, it is likely that there has been much parasite vector co-adaptation, although this might not be on-going (Rogers and Bates 2007). For example, promastigotes of *Le. infantum* have an optimum growth temperature in *P. ariasi* (Rioux *et al.* 1985) that lies within the temperature range of the natural resting sites of this vector (Ready 2008, 2013). It is not known if this parasite has recently adapted to these relatively cool sandfly resting sites, if it has manipulated the sandfly to change its resting sites or, perhaps more likely, is transmitted only in those parts of the sandfly's range that provide a suitable thermal environment. Co-adaptation need not involve co-cladogenesis or co-speciation of *Leishmania* and sandflies (Ready 2011), and the likelihood of this occurring diminishes when there is genetic exchange between vectors (Araki *et al.* 2009) and even parasites (Akopyants *et al.* 2009). Recently, hybrids of *Le. infantum* and *Le. major* have been isolated in Portugal (Ravel *et al.* 2006), and these are able to infect *P. papatasi* because they

have the *Le. major*-specific LPGs that permit midgut binding (Volf *et al.* 2007). Understanding the compatibility of parasite-vector combinations – partly by identifying permissive and specific vectors (Volf and Myskova 2007) – is fundamental for investigating *Leishmania* manipulation of sandfly feeding behaviour, but there are still many unknowns. The focus has been on the galectin-LPG binding that is characteristic of the specific vector *P. papatasi*. However, this interaction is 'necessary but not sufficient' for the survival of *Le. major* in *P. papatasi* (Dobson *et al.* 2010).

The identification of *Leishmania* manipulation of other sandfly behavioural traits will be complicated by the complexity of the organismal interactions. *Leishmania* constitutes just part of the microbiota of the sandfly midgut, which can contain competing bacteria (Dillon *et al.* 1996), to which the sandfly can react with catalases detrimental to *Leishmania* (Diaz-Albiter *et al.* 2011), and chemicals that change locally according to the preferred carbohydrates taken from aphid honey dew (Killick-Kendrick and Killick-Kendrick 1987) and plants (Junnila *et al.* 2011). Some arboviruses are specifically associated with sandflies (Dépaquit *et al.* 2010) and they might manipulate sandfly behaviour more than *Leishmania*, especially if they have a higher prevalence. Sandfly saliva can exacerbate leishmaniasis lesion development or provide some protection (Collin *et al.* 2009) but, in mouse models, it can have an antagonistic effect with fPPG rather than a synergistic one (Rogers *et al.* 2004). Salivary peptides need not display recent adaptive selection (Mahamdallie and Ready 2012).

What are the behavioural traits that are not only likely to be manipulated by *Leishmania* but also amenable to experimental investigation? The physical response of the mammalian host plays an important role in disturbing biting flies (Hurd 2003), including *L. longipalpis* feeding on experimental mice when sandfly blood feeding persistence enhances transmission (Rogers and Bates 2007). This sandfly forms blood feeding aggregations on hosts (Kelly and Dye 1996) and these can be cooperative with respect to the effects of saliva on shortening the duration of the blood meal and the quantity of blood ingested (Tripet *et al.* 2009). There are fitness implications, with long blood meals increasing the risks of hosts killing flies and larger blood meals leading to greater sandfly fecundity. To this set of interactions could be added the effects of fPPG from infected flies. The importance of fPPG will clearly depend on natural rates of infection with *Leishmania*. These are often low for *L. longipalpis* (Rogers and Bates 2007), which is even true for the vector of AVL, *P. argentipes* (Bern *et al.* 2010). A low infection rate in *L. longipalpis* might result in little selection against fPPG-regurgitating sandflies, even if they cause local disturbances of feeding aggregations because of their persistence and multiple blood feeding.

Conclusions: new ways of controlling leishmaniasis transmission

Some of the recent findings mentioned in this review lead directly to new ways of controlling leishmaniasis transmission. For example, the PSG produced by *Le. mexicana* or a chemically defined synthetic glycovaccine containing the glycans found in *Le. mexicana* PSG were found to provide protection against challenge by the bite of experimentally infected *L. longipalpis* (Rogers *et al.* 2006). However, most of the new knowledge of *Leishmani*a manipulation of sandflies has yet to be exploited for controlling the transmission of leishmaniasis. It offers the possibility of control using reduced amounts of insecticides and repellents (Ready 2010, WHO 2012) and avoiding the culling of reservoir hosts (Quinnell and Courtenay 2009). However, there will surely be many challenges to applying the new knowledge to achieve more ecologically friendly methods of effective integrated control (Ready 2013). Realism, not pessimism, is required to meet these challenges, most of which will probably arise because of the complexities of the behavioural interactions between sandflies and between sandflies and infecting *Leishmania*. For example, the male pheromones that attract female *L. longipalpis* to lekking sites on vertebrate hosts can

be synthesised and might be distributed peridomestically in NW ZVL foci in order to reduce transmission to humans (Bray *et al.* 2010), but such interventions or similar ones, for example zooprophylaxis using chickens, are only likely to be effective if the possibilities of unintentionally increasing human biting rates are fully investigated (Kelly and Dye 1996). Similarly, modelling is required to investigate how manipulating the aggregation behaviour of *L. longipalpis* (Tripet *et al.* 2009) will affect mammalian host exposure to fPPG and sandfly saliva. The question is whether the parasite manipulation of *L. longipalpis* and other sandfly vectors can itself be artificially manipulated for the purposes of prophylaxis. The achievement of this will require an awareness of the possibilities of unintentionally exacerbating disease (Rogers *et al.* 2008; Collin *et al.* 2009).

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Strategic issues concerning vector-parasite interactions

10. Modelling the control of mosquito-borne diseases

Ace North and Penelope Hancock

Abstract

We focus on strategies for controlling the epidemiology of mosquito-borne diseases that target the mosquito vector. In order to assess a particular strategy of mosquito population control, two broad issues require attention: the dynamics of the population, and the impact of the intervention on those dynamics. We describe two modelling approaches that are important tools in these respective tasks. Firstly, backwards modelling, the method of using models to interpret complex data, is a valuable means to understanding mosquito ecology. We exemplify backwards modelling with a brief review of research into how larval competition impacts on mosquito population dynamics, an important question to answer given that this density dependent process may significantly interact with some intervention strategies. Secondly, forwards modelling, whereby models are used to forecast the outcome of interacting ecological processes, allows ecological knowledge to be utilised so that the impacts of a particular strategy can be predicted. In particular, forward models allow investigation of how the performance of strategies may vary across different ecological settings. We exemplify forward modelling with an illustrative model of how climatic temperature may influence the effectiveness of a fungal biopesticide intervention. We end the chapter with a brief discussion on important future directions.

Keywords: mosquito-born disease, population dynamics, mathematical model, density-dependance, vector control, biopesticide

Introduction

While strategies to control most infectious diseases aim to target the pathogen, vector-borne diseases offer an alternative target: the vector. Mosquitoes are vectors of a number of major diseases, including malaria, dengue fever, and yellow fever (Gubler 1998).

At present chemical insecticides are the mainstay of mosquito control, delivered via insecticide treated nets (ITNs) and indoor residual spraying of insecticides (IRS). In the case of malaria, these technologies have successfully suppressed or eliminated transmission in some locations (Curtis and Mnzava 2000, Haworth 1988, Killeen *et al.* 2007), but they have been unable to eliminate malaria from the worst affected parts of tropical Africa, where control is difficult due to poverty and climatic conditions that are conducive to endemic transmission (Ferguson *et al.* 2010). A number of novel strategies are under intensive development, including biological control methods involving the introduction of bacterial and fungal infections into mosquito populations (Blanford *et al.* 2005, McMeniman *et al.* 2009, Moreira *et al.* 2009, Scholte *et al.* 2005), and the release of genetically modified mosquitoes to suppress the native population or initiate the spread of phenotypes that do not transmit disease (Burt 2003, Thomas *et al.* 2000, Ward *et al.* 2011).

As such, a various arsenal will, it is hoped, be available to future practitioners of mosquito control (Takken and Knols 2009). Clearly, in order to inform the design of control programmes in different areas to meet specific targets, there is a need to understand the impacts of each technology on the mosquito population and its capacity to transmit disease. This will require detailed knowledge of the ecology of the target mosquito population (Ferguson *et al.* 2010).

W. Takken and C.J.M. Koenraadt (eds.), *Ecology of parasite-vector interactions*, Ecology and control of vector-borne diseases 3, DOI 10.3920/978-90-8686-744-8_10, © Wageningen Academic Publishers 2013

Models have an important role to play in both qualifying and quantifying the ecological consequences of the different control measures. Two types of modelling stand out in mosquito research, denoted in this chapter as `backward' and `forward' modelling (Figure 1). In the following Section 'Understanding mosquito ecology', we discuss how backward modelling, whereby models are sought to describe and explain observed population patterns, is becoming an important approach to understanding mosquito ecology. In order to illustrate the discussion, we focus on a particular aspect of mosquito ecology: the influence of larval competition on population-level dynamics. By contrast, forward modelling, whereby models are used to predict the outcome of interaction between ecological processes, has more often been used to asses the impacts of population control strategies (Section 'Models of strategies for controlling mosquito-borne diseases'). We note that the distinction between backward modelling as a tool to understand mosquito ecology, and forward modelling to understand mosquito control, is neither fundamental nor clear-cut, but rather a useful generalisation.

Understanding mosquito ecology

Despite the importance of mosquito ecology in determining patterns of disease transmission, our understanding of the basic ecology of relevant species is fragmented at best. For example, while a good deal is known about the habitat and blood-meal preferences of the important malaria vector *Anopheles gambiae* Giles, there are few data on the dry-season ecology or the mating behaviour of this species (Jawara *et al.* 2008, Lehmann *et al.* 2010, Takken and Boëte 2003). Amassing ecological data on a particular mosquito species is, clearly, a vital step towards building a general understanding of the ecology of said species. Data in itself, however, is only useful as far as it is understood: data interpretation is equally vital. In view of the large number of ecological processes and environmental factors that may impact on mosquito populations, it is often difficult to disentangle their interacting effects from observational data. If a researcher is focussed on elucidating the roles of particular processes, experimental studies may be designed so as to greatly reduce the number of confounding factors, yet even experimental data may be hard to interpret if the processes under study are complex. Mathematical models, however, are able to show how complex processes behave and interact for any given set of underlying assumptions (Kokko 2007, Turchin 2003). As such, models are increasingly being used to help interpret mosquito data.

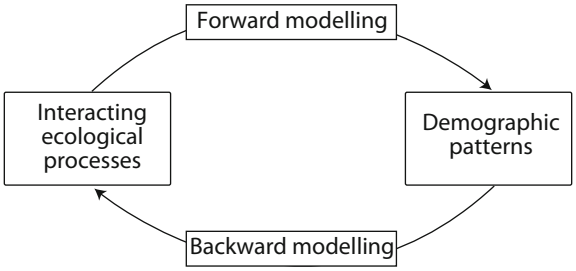

Figure 1. Two types of modelling approaches have been useful to the study of mosquito control. Forward modelling, whereby the interaction between a complex set of factors is predicted, has been particularly useful in the evaluation of mosquito control strategies. Backward modelling, on the other hand, is a method to interpret complex data and has been useful to the study of mosquito ecology.

In the remainder of this section, we exemplify how models have been used to aid the study of mosquito ecology by briefly reviewing research into a specific yet important question: what is the role of larval competition to mosquito population-level dynamics? (Dye and Hasibeder 1986, Legros *et al.* 2009, Russell *et al.* 2011b, Yang *et al.* 2008a,b). All the studies discussed employ a backward modelling approach (Figure 1), whereby data is used to parameterise one or more models. If there is more than one model, statistical tests are used to compare the performance of each model (Russell *et al.* 2011b, Yang *et al.* 2008a,b). In this way, the models act as alternative hypotheses, and the model comparison enables the hypotheses to be contested against one another, an increasingly popular approach in ecological research (Johnson and Omland 2004).

Larval competition

While it is widely acknowledged that exogenous factors, such as rainfall, play a major role in the regulation of mosquito populations (Edillo *et al.* 2004, Koenraadt *et al.* 2004), there is less certainty over the role of the density-dependent feedback that stems from larval competition (Legros *et al.* 2009). From the limited number of field studies that assess the process of population regulation in mosquito species, there is evidence that density-dependent feedback has an important influence on the population dynamics of *Aedes aegypti* L. (Dye 1984, Legros *et al.* 2009, Southwood *et al.* 1972), *Aedes vigilax* Skuse (Yang *et al.* 2008a,b) and *An. gambiae* (Russell *et al.* 2011b). Adult abundance may depend indirectly on juvenile density; for example higher larval density has been observed to prolong juvenile development time and reduce adult body size in *An. gambiae* (Gimnig *et al.* 2002, Russell *et al.* 2011b), which may reduce the population fitness (Russell *et al.* 2011b). The relative influence of larval competition and parasitism and predation of larvae on larval density is not well-quantified (Koenraadt *et al.* 2004, Service 1977).

There is strong motivation to redress this uncertainty: density dependent feedbacks may significantly impact on the success of population control measures, as discussed in the Section 'Models of strategies for controlling mosquito-borne diseases'. The studies reviewed below used very different data to asses the role of larval competition to population dynamics, broadly classed as 'Survivorship data' (Dye 1984, Legros *et al.* 2009) and 'Abundance time-series' (Dye 1984, Legros *et al.* 2009, Russell *et al.* 2011b, Yang *et al.* 2008a,b). We end this section with a brief perspective comparing the merits of the contrasting study designs.

Survivorship data

Dye (1984) fitted a model of *Ae. aegypti* population dynamics using data that measured the survivorship of cohorts during larval development, where the cohorts differed in initial density. Dye (1984) supposed that r_d, per capita density dependent mortality during the larval stage, may be described by the function:

$$r_d = \alpha N^{\beta} \tag{1}$$

where N is the clutch size (density of eggs), and α and β are free parameters to describe the form of density dependence (Figure 2). In order to fit this model to the data, Dye (1984) was forced to ignore the possibility of concurrent density independent mortality occurring in the system: the data was not sufficient to fit a model allowing both causes of mortality. In doing so, Dye (1984) was able to estimate α and β using a non-linear regression analysis.

In turn, the parameter estimates obtained by Dye (1984) have been incorporated into more complex population models of *Ae. aegypti* (Phuc *et al.* 2007, Yakob *et al.* 2008a). This story, however, should perhaps act a cautionary tale on the importance of keeping the assumptions of a model in mind when assessing its conclusions. On relaxing the assumption that all larval mortality is due to density dependent factors, Legros *et al.* (2009) show that density dependence may have been significantly overestimated by Dye (1984), which casts doubt not only on Dye's conclusions (in defence, Dye is clear about the underlying assumptions), but also on the conclusions of the subsequent modelling studies. Legros *et al.* (2009) reason that the data set used by Dye is insufficient to reliably parameterise the function of Equation 1, and therefore to characterise the role of density dependence during larval growth. The moral: while overly complex models cannot be parameterised with limited data-sets (the hazards of overfitting), the assumptions of simpler models, that are parameterised with data, must be clear and remembered when those parameters are used subsequently.

Abundance time-series

If density dependent processes are sufficiently strong to impact on population dynamics, one may expect to detect the effects of density dependence in time-series data (Hanski 1990). In view of the number of (especially environmental) factors influencing population dynamics besides density dependence, this task will inevitably be a challenging one yet, if environmental data is collected concurrently, not necessarily a hopeless one. There has been debate over the most appropriate statistical test for density dependence in time-series (Hanski 1990), although the classical approach of employing a null model (density dependence is absent) has been prevalent in the past (Yang *et al.* 2008b). More recently, the approach of model selection (or 'multi-model inference', MMI) has become increasingly popular in many fields of ecology (Johnson and Omland 2004), and the evaluation of density dependence from time-series data is no exception (Brook

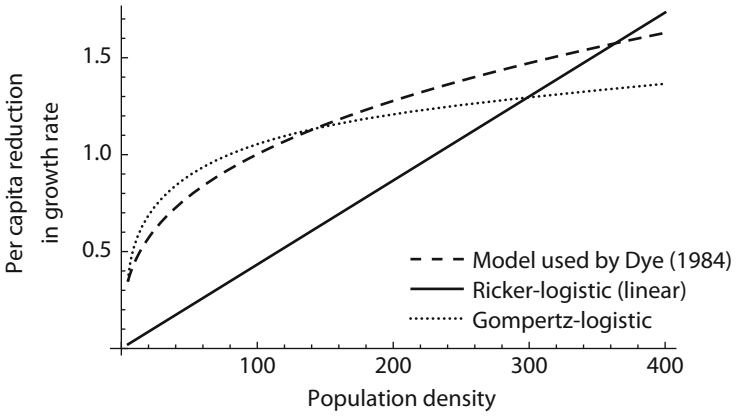

Figure 2. Different models of density dependence have been proposed for the effect of larval competition on population growth (see Equations 1-3). Empirical data have been used to compare these alternatives. The parameters are (α, β) = (0.23, 0.3) (Dye), and (r_m, K) = (1, 200) in both the Ricker-logistic and the Gompertz-logistic models: these parameters were chosen to illustrate the functional forms rather than derived from particular mosquito populations.

and Bradshaw 2006, Zeng *et al.* 1998). Unlike null model testing, model selection allows multiple hypotheses (models) to be compared simultaneously: each model is fitted from the available data, and the goodness-of-fit is compared across the models, often using selection criteria that penalise against model complexity (Johnson and Omland 2004).

Model selection has been employed to assess the relative roles of density dependent, density independent, and environmental processes in *An. gambiae s.l.* (Russell *et al.* 2011b), *Ae. vigilax* and across six tropical species (Yang *et al.* 2008a). To the best of our knowledge, these are the only published studies that have used time-series data to assess density dependence in mosquitoes. Each of the articles used maximum likelihood estimation to fit a suite of models to the respective data-sets, where the models differed in their representation of density dependent and environmental factors. The model performances were compared using Akaike's Information Criterion (AIC) (Russell *et al.* 2011b, Yang *et al.* 2008a,b) and, additionally, Bayesian Information criterion (BIC) and cross-validation (C-V) (Yang *et al.* 2008a,b).All three articles found significant evidence of density dependent feedbacks. Yang *et al.* (2008b) finds endogenous feedbacks to explain ~42% of the variation in growth rate of *Ae. vigilax*, with the remainder explained by exogenous environmental conditions, and the authors thus suggest a rule of thumb: 'negative density dependence is nearly as important as environmental conditions when predicting the abundance of mosquito populations over time'. Perhaps unsurprisingly, however, the details of how density dependence acts on the populations are much less consistent.

While Russell *et al.* (2011b) concur that density dependence is important to *An. gambiae s.l.*, the density dependent feedback component of the best model takes the Ricker-logistic form, whereby r_d, the per capita mortality rate due to density dependence during one time step (~ one generation) is linear:

$$r_d = \frac{r_m N_t}{K} \tag{2}$$

Where N_t is the population size at the start of the time step, r_m the maximal intrinsic growth rate, and K the carrying capacity. By contrast, Yang *et al.* (2008b) found the Gompertz-logistic model:

$$r_d = r_m \frac{\log N_t}{\log K} \tag{3}$$

to give an 'overwhelmingly better fit' for all six species in their study (both models were compared in both studies). This suggests density dependence acts at lower densities in the species studied by Yang *et al.* (2008b), yet the relative increase in density dependence as the population grows is less (Figure 2).

To investigate the interaction between environmental forces and density dependence, Yang *et al.* (2008a) compared (Gompertz-logistic) models for which the environmental factors impacted on, respectively, growth rate and carrying capacity. The data strongly supported the former model over the latter, suggesting that carrying capacity is relatively stable in their system while environmental variation alters the intrinsic growth rate. By contrast, the best model of *An. gambiae* linked the carrying capacity in Equation 2 to the most important environmental variable for this species (Russell *et al.* 2011b).

It is intriguing to seek the cause of these inter-specific differences, yet given that they are based on only two studies (Russell *et al.* 2011b, Yang *et al.* 2008a,b), which markedly differ in study design (for example, the time-series drastically differ in duration), it would be unwise to conclude, at this

point, that the discrepancy is due to specific divergence. Further research on this issue would clearly be useful.

Perspective

Despite the shortcomings of Dye's (1984) analysis, the approach of quantifying density dependence through larval survivorship data, and more generally through experimental manipulation, may yet give valuable insights. The experimental set-up will need to be designed carefully, however, so that the data will be sufficient to fit the intended models (Legros *et al.* 2009). One key advantage of an experimental approach over a time-series approach is that the potentially confounding factors, such as environmental variation, can be largely controlled.

Larval survivorship data will not, unfortunately, reveal effects of larval competition other than alteration to the larval mortality rate. Survivorship data may, therefore, lead to an underestimation of density dependence in species for which larval competition has other fitness effects such as a reduction in emerging adult size. Time-series data, by contrast, will subsume all density dependent processes that impact on population dynamics. In order to determine the role of specific density dependent fitness effects from time-series, however, one has to know what to look for: time-series are more useful for testing than devising hypotheses. Observational studies, that measure the phenotypic effects of density dependence, have a valuable role to play in revealing potentially important fitness effects of density dependence (Gimnig *et al.* 2002). By keeping track of female wing-length through the study period, Russell *et al.* (2011b) were able to support the hypothesis that the effects of density dependence in *Anopheles gambiae* species is mediated by this larval plasticity in growth.

In summary, it is clear that our understanding of density dependence in mosquitoes has much room for improvement, yet it is encouraging to see recent publications on the issue: it is an active field. Density dependence is qualified using mathematical models that relate the effects of density dependence to population dynamics. These models have been quantified and evaluated from both survivorship data and from time-series. Both approaches have distinct strengths, and it is thus hoped that both are pursued in future studies.

Models of strategies for controlling mosquito-borne diseases

Many models that have been developed to analyse methods of controlling mosquito vectors of human diseases adopt a forward modelling approach, whereby population dynamic models, parameterised with data on mosquito ecology, are used to predict the outcome of an intervention. A difficulty is that models can become very complicated when they attempt to provide a detailed and realistic representation of mosquito ecology. Often models are developed to selectively include only the factors that have the most obvious relevance to a specific intervention, and the questions being asked of it. In this section, we discuss how such models are used to determine how the effects of intervention on a mosquito population, and its transmission of disease, are mediated by specific demographic processes, by ecological setting, and by details of how the intervention is applied. We exemplify this discussion with a case study in which a model is developed to predict how temperature affects the success of using fungal biopesticides to control *Anopheles* mosquitoes and, in turn, the malaria they vector (Box 1).

Box 1. Case study: modelling the effect of fungal biopesticides on malaria transmission at two different temperatures.

1. Modelling adult stage structure

Blood feeding behaviour in female adult *Anopheles* mosquitoes follows a gonotrophic cycle (Klowden 2007, Lardeux *et al.* 2008). The cycle consists of a host-seeking phase, during which mosquitoes actively search for a blood meal, and a non-host-seeking stage, during which blood from a recent blood meal is digested, oocytes are developed and eggs are oviposited, after which host-seeking activity begins again. The duration of the gonotrophic cycle varies depending on the temperature conditions the mosquito experiences, with faster metabolic rates occurring at higher temperatures (Lardeux *et al.* 2008). In warmer climates *Anopheles gambiae* typically take a blood meal every 2-3 days (Quinones *et al.* 1997) where as under cooler conditions there may be over 10 days between feeds (Paaijmans *et al.* 2010).

Hancock (2009) extended classical continuous time models of mosquito population dynamics (Hancock *et al.* 2009, MacDonald 1957, Ross 1911, Smith and McKenzie 2004) to incorporate gonotrophic structure. The model divides the adult mosquito population into two classes; those that are actively searching for a (human) blood meal and those that are not host-seeking, having not yet oviposited following their recent blood meal. Host-seeking mosquitoes are assumed to find blood meals at a continuous daily rate f, and mosquitoes spend a fixed period of time T_M in the non-host-seeking stage (Figure 3). Hancock (2009) showed that the equilibrium number of adult mosquitoes in the i^{th} host-seeking stage, S^*_{Hi}, is given by:

$$S^*_{Hi} = \frac{\varepsilon f^{-(i-1)\mu T_M}}{(\mu + f)^i} \tag{4}$$

where ε is the daily rate at which adult mosquitoes are recruited and μ is the daily rate of adult mortality.

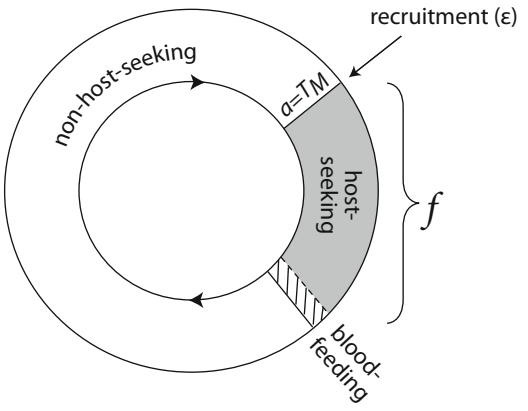

Figure 3. Diagram of the gonotrophic feeding processes represented in the model. Non-host-seeking mosquitoes resume host-seeking activity when the time since their last blood meal, a, reaches T_M days.

The model can be extended to incorporate a process of malaria transmission by the mosquito population by making assumptions about the proportion of humans that are infectious with malaria, the probability that malaria is transmitted during a bite by a mosquito on an infectious human, and the converse probability of a mosquito contracting malaria from biting an infected human. The malaria parasite is assumed to require a fixed period of incubation inside the mosquito of T_E days before it can be transmitted. With these assumptions the equilibrium number of host-seeking mosquitoes that have been infectious with malaria for *i* host-seeking periods, I^*_{Hi} can be calculated (Hancock 2009). This gives an expression for the equilibrium entomological inoculation rate (EIR), which is the number of infectious bites received per person per day (Hancock 2009):

$$EIR^* = f \sum_i I^*_{Hi} \tag{5}$$

The duration of the malaria parasite incubation period, T_E, is also strongly dependent on temperature. In warmer climates incubation can be completed in less than 10 days, whereas between 20-30 days can be required under cooler conditions (Paaijmans *et al.* 2009). As both this incubation period T_E and the time interval between blood feeds, T_M, increase as temperature decreases, these two processes can act synergistically to reduce the rate of malaria transmission in cooler climates.

2. The impact of fungal biopesticides on malaria transmission

The stage-structure implemented in this model is relevant to mosquito control interventions which act differently on host-seeking and non-host-seeking stages of the mosquito lifecycle. For a fungal biopesticide intervention that involves spraying surfaces inside houses with spores of a fungal entomopathogen, mosquitoes that are host-seeking are more likely to contract the fungus than those that are not host-seeking and away from areas of human habitation. The model assumes that host-seeking mosquitoes contract the fungal pathogen at a daily rate *F*, and so the daily probability of infection is $P_f = 1\text{-}e^{-F}$. The conservative assumption that non-host-seeking mosquitoes have zero risk of fungal infection is adopted. Experimental studies show that fungal infection causes accelerated adult mortality, with the effect becoming more pronounced with increased age of infection (Blanford *et al.* 2005). Depending on the fungal strain that is used and the dose that is applied, a wide range of age-dependent mortality patterns have been observed (Blanford *et al.* 2005, Scholte *et al.* 2003, 2006). Here, we estimate the effect of fungal infection on adult mortality using data from recent field trials of the application of *Beauvaria bassiana* (Balsamo) Vuillemin in experimental huts in Tanzania (Mnyone, unpublished data). Weibull functions describing the rate of adult mortality as a function of the age of fungal infection were fitted to the experimental data (Hancock 2009; Figure 4). In the model, these functions describe the age-dependent effect of fungal infection on adult mortality (Hancock 2009).

We consider the effect of fungal biopesticide application on the rate of malaria transmission (the daily EIR, eqn 5) for two different temperature conditions. In the first case, the temperature is warm (a constant 30 $^{\circ}$C), in which case we estimate that the duration of the non-host-seeking stage is T_M=2 days (Quinones *et al.* 1997) and the duration of malaria parasite incubation is T_E=8 days (Paaijmans *et al.* 2010). Secondly we consider a cool environment in which the temperature is a constant 20 $^{\circ}$C, for which we estimate that T_M=6 days and T_E=20 days (Paaijmans *et al.* 2010). All other model parameters are the same as in Hancock (2009) unless otherwise specified.

Figure 5 shows the effect of the fungal biopesticide on the equilibrium daily EIR for different values of the daily probability of fungal infection experienced by host-seeking mosquitoes

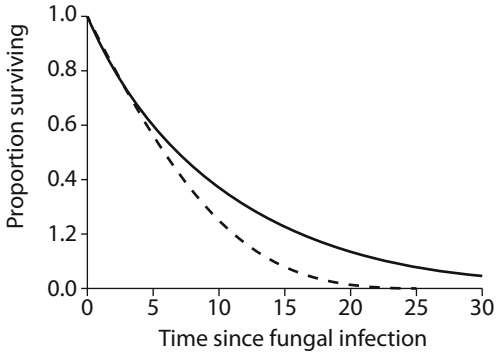

Figure 4. Model of the age-dependent effect of fungal infection on adult mortality. Mosquitoes uninfected with the fungal pathogen (solid line) and infected (dashed line) both experience natural mortality at a constant daily rate μ=0.1. Mosquitoes that are fungus-infected experience additional mortality at a rate that depends on the time since fungal infection. The additional mortality rate $M_F(u)$ *is modelled by a Weibull function of the fungal infection age* u; $M_F(u) = \beta\mu_F(\mu_F u)^{\beta-1}$, *where* $\mu_F=0.068$ *and* $\beta=2.5$ *are the Weibull rate and shape parameters respectively.*

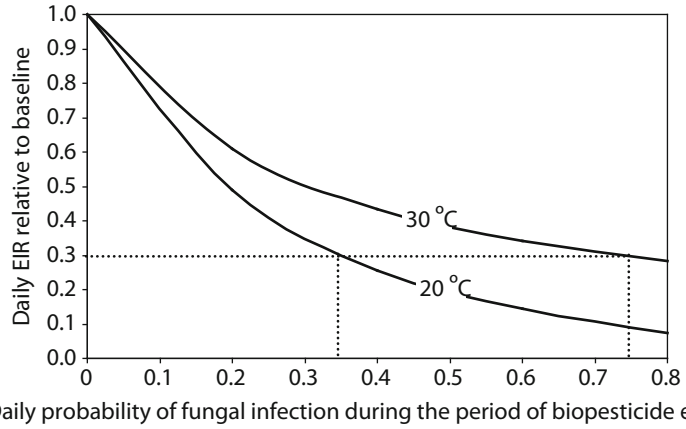

Figure 5. The equilibrium daily EIR as a proportion of the baseline EIR for different levels of the fungal biopesticide coverage. Solid lines show different values of constant temperature. Dotted lines indicate the coverage levels required to achieve a 70% reduction in the EIR for both temperatures.

(termed the 'coverage' of the biopestide). This shows that the fungal biopesticide is more efficient, having a greater proportional effect on the EIR, when the temperature is cooler. For example, if very high coverage can be achieved, the EIR is reduced by about 70% at the warmer temperature and by about 90% at the cooler temperature. It is perhaps more significant that a considerable reduction in the EIR, of about 70%, can be achieved with moderate to low biopesticide coverage (about 0.3-0.4) at 20 °C, but a similar proportional impact on the EIR would require a much higher coverage of about 0.7-0.8 at 30 °C.

The reason for the greater efficacy of the biopesticide at cooler temperatures is that there is more time for fungal infection to act when both the delay between blood feeds and the duration of malaria parasite incubation are longer. The experimental data shows that fungal infection is relatively slow to kill mosquitoes (Figure 4), and so is less effective in reducing malaria transmission when mosquitoes start taking malaria-infectious blood meals at a younger age. However, the intervention still produces a substantial decrease in the EIR at the warmer temperature, and Figure 5 indicates that the absolute reduction in the EIR will be greater at 30 °C because the baseline EIR is much higher when the temperature is warmer. An important caveat to these results is that the effect of fungal infection on mosquito mortality is also significantly affected by temperature. A study by Kikankie *et al.* (2010) measured the survival of fungus-infected mosquitoes at two different temperatures, and found that the fungus was significantly more virulent at warmer temperatures. Therefore, further investigation of how this intervention affects key aspects of mosquito demography across a range of different environmental conditions is needed to understand how malaria transmission can be impacted in different areas.

Demographic processes

The process of density-dependent larval competition, discussed in the previous section, is of central importance to certain novel strategies for controlling mosquito-borne diseases by releasing genetically modified mosquitoes. One such strategy developed to help control *Ae. aegypti* mosquitoes, which vector a number of important human diseases including dengue fever and yellow fever, involves the release of genetically modified male mosquitoes that carry a dominant genetic system that is lethal to their offspring. This strategy, known as RIDL (Phuc *et al.* 2007, Thomas *et al.* 2000), can be designed so that the offspring resulting from matings between wild females and the genetically modified males die either before starting or after completing larval development (Phuc *et al.* 2007). It is expected that the latter strategy (known as late-lethal) would be more effective that the first (known as early-lethal) in suppressing the wild mosquito population, because the doomed offspring would survive through the larval stage to compete for resources and so their death would not reduce the density-dependent competition experienced by the larval population. Models of the RIDL strategy address the question of how the suppression in adult abundance is affected by the higher density-dependent larval competition associated with the late-lethal strategy (Atkinson *et al.* 2007, Phuc *et al.* 2007). Atkinson *et al.* (2007) derive threshold conditions for the eradication of the dengue virus for the early-lethal and late-lethal strategies in terms of demographic parameters relevant to mosquito vectorial capacity. However, quantification of these thresholds requires detailed data on the relationship between the survival of larvae and their density, which is either very scarce or non-existant, as discussed previously.

Conversely, many vector control methods, both traditional and novel, target mosquitoes in the adult stage of their lifecycle, as it is the older-aged adult mosquitoes that transmit human diseases. Such strategies include IRS and ITNs. A number of novel strategies for adult mosquito control are currently being developed, motivated by the rising levels of resistance in mosquito populations to the commonly used insecticides (Ranson *et al.* 2009). In Box 1 we describe a model of a novel method of biological control of mosquito populations. Fungal biopesticides can be used to infect adult mosquitoes with a pathogenic fungus which interferes with their mobility and blood-feeding activity and reduces their lifespan (Blanford *et al.* 2005, Scholte *et al.* 2006, Scholte *et al.* 2005). The model focuses on the demography of adult *Anopheles* mosquitoes and

aims to capture aspects of the adult mosquito's biology that are important to the effect of fungal biopesticides on the capacity of these mosquito populations to transmit malaria.

Ecological setting

An important purpose of models of mosquito control strategies is to investigate how the performance of strategies may vary across different ecological settings. Mosquito demographic rates can be strongly dependent on environmental conditions; for example temperature affects developmental processes in the mosquito, including the rate of development of the juvenile stages (Pascual *et al.* 2006), the rate of adult mortality, and the frequency of blood feeding (Paaijmans *et al.* 2010). The development rate of the parasites and viruses transmitted by mosquitoes is also sensitive to temperature (Paaijmans *et al.* 2009, Watts *et al.* 1987). Spatial analyses of malaria transmission intensity have used temperature and rainfall data to characterise the epidemiology of malaria throughout Africa in terms of whether the disease is likely to be absent, epidemic or endemic in different regions (Craig *et al.* 1999). The efficacy of vector-control interventions varies depending on the environmental conditions in the area where they are applied; for example IRS has been least effective in reducing malaria transmission in endemic tropical and lowland areas (Mabaso *et al.* 2004). In our case study we investigate how the ability of fungal biopesticide interventions to reduce the rate of malaria transmission may vary in areas with different temperature conditions (Box 1).

Seasonal fluctuations in mosquito abundance in response to rainfall patterns is also an important consideration for any mosquito control strategy. Accurate and effective early warning systems are valuable in allowing interventions to be implemented in time to contain seasonal outbreaks of disease (Thomson *et al.* 2006). Interventions such as the sterile insect technique (SIT), that involve releasing laboratory-reared mosquitoes to interfere with the reproduction of the wild population, may only be successful if they are well-timed. For example, the released mosquitoes may be overwhelmed if the natural population is rapidly expanding, but if the releases occur when conditions are too dry then the introduced mosquitoes may experience high mortality, particularly as they will need to locate relatively scarce suitable habitat to find mating partners. Given that mosquito abundance can increase over several orders of magnitude during the first 2-3 weeks of the rainy season (Laneri *et al.* 2010, Lehmann *et al.* 2010), seasonality represents a major practical challenge for these interventions in some areas.

Similar issues arise with newer technologies aiming to release genetically modified mosquitoes (Deredec *et al.* 2008, Gould *et al.* 2008, Marshall *et al.* 2011). These strategies can be used to spread genes through the population that have a detrimental effect on the mosquito or on the pathogen that it transmits, lowering the vectorial capacity of the population (a process known as 'population replacement'). Often it is necessary that the frequency of the gene exceeds a certain threshold level in the population in order for it to spread, and so the strategy requires the abundance of natural population to be sufficiently low that the insect releases can attain this frequency (Deredec *et al.* 2008, Marshall *et al.* 2011). This condition also applies to novel biological control methods involving the release of mosquitoes infected with *Wolbachia* bacteria, because *Wolbachia* also only spread once their infection frequency exceeds a threshold (Hancock *et al.* 2011a,b, Hoffmann *et al.* 2011, McMeniman *et al.* 2009, Moreira *et al.* 2009, Turelli 1994, Walker *et al.* 2011). Mosquito population dynamic models that explicitly represent seasonally varying demographic rates, and the mating frequency between released and wild mosquitoes, can be used to explore the effects of the timing of releases on the number of introduced insects required for spread to occur in a seasonal environment (Hancock *et al.* 2011a,b). The results emphasise that an ability to understand

and predict seasonal variation in mosquito population dynamics can be vital to the success of control strategies that involve deliberate mosquito releases.

Future directions

With growing attention being given to the control of mosquito vectors, it is clear that models will be increasingly called upon to build a theory of mosquito dynamics, and to investigate the impacts of specific control measures. Both the modelling approaches discussed in this chapter – backward modelling to interpret complex mosquito data, and forward modelling to predict the consequences of specific interventions – are currently being applied, yet this is a young field of research. We end this chapter with a brief comment on three important, yet under-studied, aspects of mosquito dynamics where modelling attention is required: evolution, spatial structure, and community effects.

Evolution

Both mosquito vectors, and the pathogens they transmit, have unfortunately proven themselves remarkably adaptable in the face of certain control measures. *An. gambiae s.l.*, for example, have evolved to become less susceptible to certain insecticides, both through physiological changes (John *et al.* 2008) and behavioural changes (Pates and Curtis 2005), in only a few decades at most. Genomic study is proving useful to resolving the molecular mechanisms of resistance evolution (Hemingway *et al.* 2004, Weill *et al.* 2003), yet genomic insight alone cannot reveal the spatio-temporal dynamics by which resistance spreads among populations. Population genetic models are increasingly being used to study how intervention strategies are affected by evolutionary feedbacks in pest control (Onstad and Guse 2008), yet fewer models have been specifically tailored to mosquitoes (but see Koella *et al.* 2009, Read *et al.* 2009). To predict the conditions necessary for novel intervention strategies to be successful, it is clearly necessary to account for the potentially disruptive process of resistance evolution.

Spatial structure

In recent decades, questions of population spatial structure have become increasingly central to ecological and evolutionary research (Borcard *et al.* 2004, Hanski 1999, Thomas and Kunin 1999). The spatio-temporal dynamics of mosquito populations will be critical to the success of high-tech control strategies (Knols and Scott 2003). For instance, transgenic technologies rely on gene-flow, and so in order to make robust predictions of the conditions necessary for transgenic technologies to succeed, it is imperative to use models that account for processes, such as dispersal, that mediate gene-flow (Yakob *et al.* 2008a,b). Thanks in part to improvements in mapping technology, field biologists are increasingly recording spatial as well as temporal details of mosquito populations (e.g. Eisen and Lozano-Fuentes 2009, Fillinger and Lindsay 2006, Majambere *et al.* 2010). Spatial realism is notorious for the extent to which it complicates population models, however, and so it is perhaps unsurprising that few attempts have been made to compare spatio-temporal data to spatially realistic population models (though see Xu *et al.* 2010). Somewhat more effort has been applied to developing spatially realistic forward models in order to ask how spatial complications might affect the operation of novel control measures (Yakob and Bonsall 2009, Yakob *et al.* 2008a,b), yet our understanding of the spatial dynamics of mosquitoes clearly still has a long way to go.

Wider ecological impacts

Predicting how mosquito control will impact upon interacting species is a difficult task, yet community effects are to be expected for those interventions which strongly impact on mosquito populations. To dismiss these effects invites the possibility of unexpected outcomes of intervention, including failure (e.g. in the control of pest mammals from New Zealand forests, Tompkins and Veltman 2006). It has been suggested that even the eradication of mosquito species would have few wider ecological impacts (Fang 2010), yet this view has been staunchly criticised (Smith 2010), and it is clear that more research is needed. Although empirical study (especially manipulative experimental study) will inevitably underpin this research, models are also required. Models will be essential to interpreting multi-species data, and predicting the consequences of inter-specific interactions. The framework of community modules may be particularly useful to the study of how particular interventions might influence species interactions (Gilman *et al.* 2010). The interaction between congeneric mosquito species, which share similar ecological niches yet may respond to control measures differently, deserves particularly close attention. For example, the congeneric *An. gambiae s.l.* and *An. funestus* Giles mosquitoes differ in the extent to which they have evolved resistance to insecticide-treated nets and indoor residual spraying (Russell *et al.* 2011a). Since the niches of these species overlap, it is to be expected that the responses of each species to intervention will influence one another.

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11. Heterogeneity in malaria transmission: underlying factors and implications for disease control

Teun Bousema and Amrish Baidjoe

Abstract

In this chapter, we describe the current evidence for the existence of hotspots of malaria transmission. Heterogeneity is a common element of many infectious diseases, whereby infection and disease are concentrated in a small proportion of individuals and not distributed evenly across the population. In malaria this heterogeneity is manifested as small groups of households, or hotspots, within malaria endemic communities that are at a substantially increased risk of malaria transmission compared to surrounding households. These hotspots exist in all transmission settings, but are most easily detected at low transmission. The ecological, human and entomological factors that influence the occurrence of hotspots are currently not fully understood. Human genetic components are strongly related to the risk of (severe) clinical disease but their role in determining the location and intensity of hotspots remains uncertain. The roles of factors related to mosquito exposure are more apparent in defining geographical patterns in transmission intensity. The importance for malaria control and elimination lies in the fact that hotspots maintain transmission in low transmission seasons, representing the source of infection to the general community when vector densities increase. Hotspots of malaria transmission thereby form small geographical areas where malaria transmission is more intense and from where malaria may spread to the remainder of the community. Interventions targeted to hotspots of malaria transmission hold promise to reduce transmission intensity in the community as a whole. Before hotspots can be targeted, operationally attractive approaches to identify them need to be defined. Some of these approaches are described in this chapter together with a tool-box for hotspot-targeted interventions.

Keywords: *Plasmodium* falciparum, heterogeneity, hotspots, elimination, transmission, Anopheles

Spatial variation in malaria incidence

The occurrence of clinical malaria attacks is not equally distributed in space in time. In many endemic regions, malaria incidence shows striking spatio-temporal variation. The temporal variation in malaria exposure is well described for areas of seasonal malaria transmission. The extent of seasonality differs between regions and can be quantified as the proportion of all malaria episodes occurring in the peak transmission season. Marked seasonality can be defined as a transmission pattern in which more than 75% of malaria episodes occur in 6 or fewer months (Roca-Feltrer *et al.* 2009); in some areas ~90% of all malaria episodes may occur in a period as short as 3 months (Giha *et al.* 2000). Malaria transmission is practically absent in months when drought and/or temperatures are less suitable for mosquito propagation or the development of malaria parasites inside their mosquito vectors (Giha *et al.* 2000, John *et al.* 2009, Shililu *et al.* 2003).

In addition to this variation in time, geographical variation can be very pronounced. Spatial variation in malaria exposure exists between neighbouring villages (Bejon *et al.* 2010, Bousema *et al.* 2007, Kreuels *et al.* 2008) or sub-villages (Bousema *et al.* 2010a, Drakeley *et al.* 2003) and even between households in the same village (Bejon *et al.* 2010, Bousema *et al.* 2010a, Gaudart *et al.* 2006, Ghebreyesus *et al.* 1999). In longitudinal studies where the incidence of clinical malaria

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attacks is quantified at an individual level, it is commonly observed that some individuals can remain malaria-free for more than one year while others experience multiple malaria attacks during the same period (Bousema *et al.* 2010a, Clark *et al.* 2008, Mwangi *et al.* 2008). In an area of 16 km^2 exposed to low and unstable malaria transmission in the highlands of Kenya, small geographical areas were identified where malaria risk was up to 40-fold higher than elsewhere (Ernst *et al.* 2006). In studies conducted in areas of low to moderate endemicity in Uganda and Tanzania, 47-69% of children remained malaria free during a period of >20 months while others experienced up to 9-14 malaria attacks (Bousema *et al.* 2010a, Clark *et al.* 2008).

It has long been assumed that this heterogeneity is absent or at least far less evident in areas of higher endemicity (Carter *et al.* 2000). It is, however, likely that even in highly endemic regions considerable variation in the exposure to malaria infected mosquitoes exists (Carter *et al.* 2000, Kreuels *et al.* 2008, Trape *et al.* 2002). This heterogeneity in exposure may not lead to easily detectable variation in malaria incidence in these regions, because many infections in high endemic regions do not cause clinical symptoms (Males *et al.* 2008, Proietti *et al.* 2011) and even the individuals with the lowest relative exposure may experience at least one malaria episode in a year (Trape *et al.* 2002). As a consequence, variation in malaria exposure within these regions may remain undetected. Despite this, micro-epidemiological variations in disease risk can be detected in areas with an entomological inoculation rate (EIR) of >100 infected bites per person per year (ibpy) if studies prospectively quantify malaria incidence, or the incidence of malaria infections regardless of symptoms (Bousema *et al.* 2011, Gaudart *et al.* 2006), and link incidence to individual geo-located households. In a region of intense malaria transmission in Ghana (EIR ~400 ibpy), some children experienced malaria attacks at a rate that was five-fold higher than the village average while one-third of children remained malaria-free over a period of 21 months (Kreuels *et al.* 2008). Our current understanding is that spatial variation in malaria exposure is present across all levels of transmission intensity but is more easily recognised at lower endemicity.

Defining hotspots of malaria transmission

Global trends of reducing malaria transmission intensity (Barnes *et al.* 2009, Bhattarai *et al.* 2007, Ceesay *et al.* 2008, Kleinschmidt *et al.* 2009, O'Meara *et al.* 2008), have changed the epidemiology of malaria. These changes have uncovered heterogeneity in disease transmission in areas that were previously exposed to intense and apparently homogeneous malaria transmission. In these areas, small localities of intense transmission intensity can exist (or persist) in regions with a lower average level of malaria exposure (Bejon *et al.* 2010, Bousema *et al.* 2010a). These findings have fuelled the academic and public health interest in defining hotspots of malaria transmission intensity (Dolgin 2010). For this, it is essential to define what is meant by *hotspots of malaria transmission*. The current literature is inconsistent; entire countries or islands are sometimes classified as malaria hotspots (Singh *et al.* 2009, Toty *et al.* 2010), while some studies reserve the term hotspots of malaria transmission for small geographical areas that form part of a larger endemic region (Bejon *et al.* 2010, 2011a, Bousema *et al.* 2010a). Two related but distinct geographical elements in malaria transmission should be separated:

1. The World Health Organization definition of a *focus of malaria transmission* is a defined and circumscribed locality situated in a currently or former malarious area and containing the continuous or intermittent epidemiological factors necessary for malaria transmission. Foci of malaria transmission can be classified as residual active, residual non-active, cleared up, new potential, new active, endemic or pseudo foci (World Health 2007). In more academic terms, an active focus of malaria transmission is a geographical area that supports malaria transmission, where the local *Anopheles* population sustains R_0, the average number of secondary cases

arising in a susceptible population as a result of a single human case over the course of their malaria infection, to a level >1 (Bousema *et al.* 2010a, Carter *et al.* 2000). A mosquito breeding site forms the centre of a focus of malaria transmission. The size of the focus of malaria transmission depends on the maximum effective dispersal range of vector mosquitoes; the border is the location furthest away from the breeding site where malaria is still supported by this breeding site.

2. A *hotspot of malaria transmission* is defined as a geographical part of a focus of malaria transmission where malaria transmission exceeds the average level. Micro-epidemiological conditions for malaria transmission are favourable in a hotspot of malaria transmission, resulting in R_0 estimates that exceed the average for the focus of malaria transmission. The size of a hotspot of malaria transmission is smaller than the maximum dispersal range of vector mosquitoes; its borders are defined by the distance from the centre of the hotspot where transmission intensity is no longer (statistically significantly) higher than the average for the focus of malaria transmission (Bejon *et al.* 2010, Bousema *et al.* 2010a).

Human factors and heterogeneity in malaria transmission

The occurrence of clinical malaria and, to a lesser extent, asymptomatic infection is influenced by innate and acquired protective responses. Several human genetic polymorphisms have been identified that have been associated with protection against (severe) clinical malaria An additional aspect of human genetic polymorphisms that is receiving increasing attention is their potential impact on gametocyte carriage and the transmission of malaria to mosquitoes (Gouagna *et al.* 2010, Robert *et al.* 1996). This results in a double interest in human genetic polymorphisms in the context of heterogeneity in malaria transmission: they may explain part of the variation in malaria incidence and may also result in differences in malaria transmission potential in the human population. Their impact on malaria incidence may influence the accuracy of detecting hotspots of malaria transmission (see paragraph on operational approaches to detecting hotspots of malaria transmission), their impact on transmission potential may contribute to the formation of hotspots of malaria transmission.

Genetic factors related to human susceptibility to malaria infection and clinical malaria

The malaria parasite depends on human red blood cells (RBC) for shelter and the provision of nutrients for the duration of its infection in humans. It is therefore logical that it is sensitive to variations in RBCs. Over one hundred RBC-related genetic polymorphisms have been described, several of which have well described effects on the erythrocyte phenotype (Min-Oo and Gros 2005). For haemoglobin alterations affecting the β-chains (e.g. HbS, HbC and HbE) or conditions in which the balance between α and β chains is altered (e.g. α- and β-thalassaemia), there is sufficient evidence for a role in protection against (severe) malaria. The same is true for alterations in levels of a key enzyme in red blood cells, glucose-6-phosphate deficiency, and structural alterations leading to physical structural changes on the RBC membrane like the Duffy blood group (Kwiatkowski 2005). With the exception of the Duffy blood group, none of these polymorphisms seem to protect against initial infection with malaria but rather convey ways to keep the infection controlled and prevent progression to high density infections or severe disease. Although these genetic polymorphisms have direct beneficial effects for the human host, their epidemiological consequences for malaria transmission may not necessarily be equally beneficial.

Haemoglobinopathies: haemoglobin variants S, C and E

Haemoglobin (Hb) is a tetrameric molecule which comprises one of the main structural and functional elements of erythrocytes. It consists of two α-chains and two β-chains. In an altered form of haemoglobin, which is described as haemoglobin S (HbS) a mutation occurs which leads to the modification of one or both β-chains. Heterozygote carriers (HbAS) have one normal and one altered β-chain. Their red blood cells function relatively normal. This contrasts with RBCs of homozygous individuals (HbSS) in whom both β-chains are abnormal; HbSS cells assume a typical sickle shape under low oxygen conditions which leads to increased cell lysis and obstruction in the micro-vascular system. In countries where advanced medical care lacks HbSS homozygote individuals often die during early childhood. HbAS does not have this detrimental effect but protects against severe clinical consequences of malaria infection, but not against infection itself (Williams 2006). HbAS individuals may have a 90% lower risk of severe and lethal malaria compared to normal (HbAA) individuals (Aidoo *et al.* 2002, Williams *et al.* 2005b). Infected HbAS cells, sickle at a higher frequency compared to uninfected cells; sickle cells are known to be cleared in the spleen at higher frequencies. This results in a direct reduction of parasite densities and may also confer enhanced antigen presentation in the spleen, resulting in an improved acquisition of immune responses (Williams *et al.* 2005a). This immune component is supported by findings that the extent of the protective effect conferred by HbAS increases with age (Williams *et al.* 2005a). Sickle haemoglobin is also described to have a negative effect on the cytoadherence of RBCs infected with *Plasmodium falciparum*. This seems to correlate with an altered display of *P. falciparum* erythrocyte membrane protein-1 (Pf-EMP-1), a protein of great significance in terms of virulence and the adherence of infected RBC's to micro vascular veins (Cholera *et al.* 2008).

Haemoglobin C and E are both alternations in the β-globin chain but affect the erythrocytes in different ways. HbC is mainly found in West Africa and in higher frequencies in specific parts of West Africa like Ghana and Burkina Faso. The protective effect of HbC can especially be observed in HbCC homozygotes (Williams 2006) and may be related to a reduced ability of *P. falciparum* parasites to grow and multiply in these variant RBCs (Fairhurst *et al.* 2003, Olson and Nagel 1986, Williams 2006); in HbAC cells parasite growth may be at the same level as normal cells (Hutagalung *et al.* 1999). An alternative mechanism for the protective effect of HbC may be a reduced expression of *P*f-EMP-1, resulting in reduced cytoadherence of infected cells and a lower risk of severe (but not of uncomplicated) malaria (Fairhurst *et al.* 2003, Olson and Nagel 1986, Williams 2006).

Haemoglobin E is also associated with a reduced risk of severe malaria (Williams 2006). There is some evidence of reduced parasite growth in cells in both HbAE and HbEE individuals; both cell types also seem to be phagocytosed at higher frequencies when infected (Chotivanich *et al.* 2002). Similar to HbCC, HbEE is relatively benign and it is possible that selection favours both homo- and heterozygotes (Williams 2006).

Haemoglobinopathies: thalassaemias

Thalassaemias are polymorphisms resulting in an imbalance in the synthesis of α and β-globin chains of the haemoglobin molecules. α-thalassaemia is commonly found in regions of sub-Saharan Africa and Asia where malaria is or has been endemic. α-thalassaemia results from a deletion of one or more α-globulin genes. The clinical consequences depend on how many genes are still operational and how severe the imbalance between α and β globulin is. Absence of all 4 genes results in stillborns and is therefore not observed in malaria-endemic populations. Deletion of one or two of the genes may result in lower haemoglobin levels (Veenemans *et al.* 2008).

α-thalassaemia is associated with protection against severe anaemia during asymptomatic malaria infections (Veenemans *et al.* 2008), protection against severe disease but not against asymptomatic infection (Williams 2006) and probably not against uncomplicated disease (Wambua *et al.* 2006). There seems to be a strong correlation with age dependant factors determining to what degree α-thalassaemia offers protection against malaria (Mockenhaupt *et al.* 2004, Veenemans *et al.* 2011).

β-thalassaemia is associated with a lower parasite density but not prevalence (Willcox *et al.* 1983) and is more prevalent in the Mediterranean and Middle East than in sub-Saharan Africa. When infected with malaria parasites, β-thalassaemic cells show a reduced parasite growth *in vitro* when exposed to oxidant stress. Individuals with a single a-thalassaemic gene deletion seem to support parasite growth at normal rates; there is some evidence that infected thalassaemic cells to show enhanced antigen presentation (Williams *et al.* 2005c), suggesting an immune component in thalassaemia-associated protection.

RBC enzymes: glucose-6-phosphate dehydrogenase deficiency

Since erythrocytes lack nuclei and active translation machinery they are greatly dependent on some key long lived enzymes to create and maintain an appropriate environment for the cell to exhibit its function. Glucose-6-phosphate dehydrogenase (G6PD) is one of these enzymes; it metabolizes glucose through the pentose phosphate pathway and plays a key role in synthesizing NADPH. The gene coding for this enzyme is located on the X-chromosome and therefore autosomal; explaining why the more severe forms G6PD are mostly found in males in whom a mutation in a single gene results in a less efficient or completely deficient enzyme. Different mutations are responsible for varying degrees of G6PD deficiency across the globe (Ruwende *et al.* 1995), the most common African variant resulting in less severe G6PD deficiency than the Mediterranean variant (Tripathy and Reddy 2007).

The protection against malaria conferred by G6PD deficiency involves the early phagocytosis of infected RBCs. G6PD-deficient infected RBCs are phagocytized more efficiently than infected normal cells through a mechanism that may involve human immune components and the fact that they are more prone to oxidative stress. (Cappadoro *et al.* 1998) This results in a similar risk of infection while the time to reach densities that cause symptomatic or severe malaria would be longer for G6PD deficient individuals (Missinou *et al.* 2003).

Membrane proteins: ovalocytosis

Ovalocytosis is a disorder which affects the cytoskeleton of the erythrocyte. The typical round shape of RBCs is changed to a more oval shape. This condition is predominantly found in South East Asia; and although the condition is rare in most regions, it can be found in up to 15% of the population of some Asian countries. Ovalocytosis is associated with a more rigid RBC and an increased RBC adherence to endothelium receptors that are not present in the vascular endothelium of the brain (Cortes *et al.* 2005). Ovalocytosis has also been associated with resistance against invasion by some, but not all, parasite lines *in vitro* (Cortes *et al.* 2004). Ovalocytosis is associated with a reduced risk to the development of severe (cerebral) malaria (Allen *et al.* 1999, Cortes *et al.* 2005) but not against asymptomatic infection (Genton *et al.* 1995, Williams 2006).

Membrane proteins: Duffy blood group negativity

Lack of the Duffy antigen or Duffy blood group negativity, is associated with protection against *Plasmodium vivax*. This parasite is largely absent in much of sub-Saharan African countries while prevalent in Asia and South America. This geographical pattern has largely been attributed to a single nucleotide polymorphism leading to the absence of the Duffy binding protein from RBCs in most African populations. This Duffy binding protein was long thought to be absolutely essential for the binding and entering of *P. vivax* merozoites of RBCs. In more recent years, transmission of *P. vivax* has been reported in Duffy negative individuals (Menard *et al.* 2010, Ryan *et al.* 2006). This would indicate that the parasite has found an alternative pathway to invade red blood cells within individuals negative for the Duffy antigen (Mendes *et al.* 2011). Nevertheless, the Duffy antigen is a striking illustration of how human genetic polymorphisms can shape the geographical map of malaria transmission.

Other genetic related factors

The correlation between genetically defined RBC polymorphisms and malaria has fuelled ideas around the burden of disease and selective pressure. Host gene polymorphisms in relation with malaria may not be restricted to those affecting RBCs. Cohort studies in Kenya indicated that the genetic contribution to variability of malaria incidence is well beyond that explained by the anticipated effects of the haemoglobinopathies alone; genetic and unidentified household factors may each account for around one quarter of the total variability in malaria incidence (Mackinnon *et al.* 2005). Associations with severe malaria and high parasitaemia have also been found with human leukocyte antigens (HLA), a highly polymorphic family of molecules that play a crucial role in immune responses (Hill *et al.* 1992). Also other specific chromosomal regions contributing to the hosts immune response have been identified (Abel *et al.* 1992, Garcia *et al.* 1998). Differences have been found in populations of same ancestral origin but living at higher altitudes where malaria was not endemic (Terrenato *et al.* 1988). Genome wide linkage and association studies have shown several associations with genetic immune-related traits and host responses to malaria infections (Griffiths *et al.* 2005). All these combined findings prove that malaria parasites have imposed a strong selective pressure on the human genome in former and current malaria endemic regions. The list of genetic factors associated with (severe) clinical malaria is becoming longer and as more results from genome wide linkage and association studies become available, the complex pathophysiology of malaria is likely to be progressively revealed (Terrenato *et al.* 1988).

Human genetic factors related to human infectiousness to mosquitoes

There are several ways in which human genetic polymorphisms may influence transmission potential. Reductions in the risk of infection with malaria parasites or the density of malaria parasites will plausibly result in a reduction in transmission potential (Bousema and Drakeley 2011) while a longer duration of infections will in turn increase transmission potential. These conflicting possible outcomes warn against strong conclusions based on cross-sectional data that largely miss effects that become apparent with time.

There is little evidence for a reduced risk of malaria infection associated with any of the above described polymorphisms (with the exception of Duffy antigen); instead they may lower parasite density or slow parasite growth which may translate in a longer duration of asymptomatic infections. This will increase the development and duration of gametocyte carriage, which seems

to be supported by the suggestion that there is a significant human genetic contribution to gametocyte carriage in asymptomatic but not in symptomatic infections (Lawaly *et al.* 2010).

There are several indications that human genetic factors may influence gametocyte carriage and malaria transmission. Differences in gametocyte carriage were observed between tribes in West Africa that could not be explained by innate differences in immunity against asexual parasites (Paganotti *et al.* 2006). Most detailed studies on human genetic polymorphisms in relation to malaria transmission potential have focused on HbC and HbS. HbAS was associated with increased gametocyte carriage and increased density of gametocytes (Lawaly *et al.* 2010) and with an increased transmission of malaria to mosquitoes (Robert *et al.* 1996). In line with this, Gouagna and colleagues observed an association between the protective HbC and HbS genotypes and increased transmission to mosquitoes, an effect estimated as up to twofold in *in vivo* and fourfold in *ex vivo* studies (Gouagna *et al.* 2010). It was hypothesized that HbS and HbC might promote sexual differentiation, reduce human transmission blocking immune responses or increase gametocyte carriage as a result of the longer duration of parasite carriage in these individuals (Gouagna *et al.* 2010).

One longitudinal study that tried to determine a direct link between α-thalassaemia and gametocyte carriage or density failed to show a significant impact (Lawaly *et al.* 2010). Gametocyte rates were reported to be lower in children carrying the β-thalassaemia trait although the cause for this association remains to be established (Willcox *et al.* 1983). In summary, data are very limited but for HbC and S there is some evidence that the individual protection conferred by these haemoglobinopathies comes at the epidemiological cost of a higher transmission potential to the wider community.

Human genetic factors and hotspots of malaria transmission

Genetic factors that render individuals more or less susceptible to malaria infection or clinical malaria attacks can strongly influence intra-individual differences in malaria incidence. If human genetic factors cluster geographically, i.e. in compounds or villages, this clustering may be epidemiologically relevant for malaria transmission. Using the terminology of foci and hotspots of malaria transmission: neighbouring villages may differ in genetic background (Dolo *et al.* 2005) and hence their risk of malaria (i.e. contribute to the intensity of transmission in a focus of malaria transmission). In some exceptional areas, individuals who are genetically more prone to produce gametocytes during their infection may cluster geographically in such a way that they infect mosquitoes which subsequently spread the infection to populations with a different genetic make-up. Human genetic components could therefore contribute to the formation of a hotspot of malaria transmission. This has been suggested for areas in Burkina Faso where the susceptibility for malaria, and potentially a different infectiousness of different tribes to mosquitoes, has been described (Dolo *et al.* 2005, Paganotti *et al.* 2006). This scenario may prove to be less exceptional once genetic elements determining the human transmission potential are identified at household or family level and if local 'super spreaders', people who are disproportionally infectious to mosquitoes (Bousema and Drakeley 2011, Lawaly *et al.* 2010), can be identified. In this respect, the authors believe there will be an important role for human genetic factors that determine their attractiveness to mosquitoes; these factors may explain heterogeneity in malaria exposure in villages where all known risk factors for malaria are apparently homogeneously distributed. Until these factors are revealed, most studies on heterogeneity in malaria transmission justifiably focus on factors related to spatial variation in mosquito exposure.

Heterogeneity in mosquito exposure

Factors determining spatial heterogeneity in mosquito exposure

Given the nature of malaria transmission, it is unsurprising that heterogeneity in malaria incidence has long been associated with the vicinity of mosquito breeding sites. Observations from ancient Egypt and Greece already noted the association between fevers and wet ground and anti-malaria regulations in Italy in the early nineteenth century required that irrigated land had to be at least 500 meters away from general housing and at least 8 kilometres from the capital of a kingdom (Carter *et al.* 2000, Watson 1949). More recent studies described an association between malaria incidence and the distance to the forest (Kreuels *et al.* 2008), river (Lindsay *et al.* 1993b, Oesterholt *et al.* 2006), water body (Bousema *et al.* 2010a, Ghebreyesus *et al.* 1999) or confirmed *Anopheles* breeding site (Bousema *et al.* 2010a, Thomas and Lindsay 2000). The strength of these associations will depend on the productivity of breeding sites and the effective mosquito dispersal range. The productivity of mosquito breeding sites is variable and is influenced by factors including the type (Bogh *et al.* 2003, Edillo *et al.* 2004, Fillinger *et al.* 2009b, Majambere *et al.* 2008, Mutuku *et al.* 2006), size (Majambere *et al.* 2008) and stability of habitats (Mutuku *et al.* 2006), temperature (Edillo *et al.* 2004, Kirby and Lindsay 2009), rainfall (Paaijmans *et al.* 2007), vegetation (Bogh *et al.* 2003, Fillinger *et al.* 2009b, Majambere *et al.* 2008), salinity (Bogh *et al.* 2003), presence of larvae of other mosquito species (Majambere *et al.* 2008) and other micro-environmental characteristics. The presence of water in a potential focus of malaria transmission therefore does not automatically translate in an epidemiologically important source of malaria vectors. Similarly, the apparent absence of evident water bodies does not exclude the presence of a source of mosquito emergence. This was recently illustrated by the association of hotspots of malaria transmission with soil moisture content (Bejon *et al.* 2010) despite an apparent homogeneous ecology (Bejon *et al.* 2010, Dolgin 2010). Obviously, findings of higher mosquito abundance in endemic areas do not equal increased malaria transmission intensity; in northern Tanzania a highly significant cluster of higher mosquito exposure was not associated with a higher malaria incidence (Bousema *et al.* 2010a).

The effective mosquito dispersal range from breeding sites will depend on their localisation in relation to potential blood meal sources. If humans are the primary source of blood meals for mosquitoes, the dispersal of mosquitoes from their breeding sites will strongly depend on the human population density in the area surrounding this breeding site. The *Anopheles* dispersal range may generally be less than 1 km in densely populated areas (Carter *et al.* 2000, Manga *et al.* 1993, Midega *et al.* 2007, Trape *et al.* 1992) but low population densities motivate mosquitoes to extend their flying range in search of a blood meal, potentially leading to ranges of ≥2 kilometres (Carter *et al.* 2000, Ejerctto and Urbino 1951, Lindsay *et al.* 1995). In densely populated areas, there can be a strong gradient in relative mosquito exposure and distance to a (potential) breeding site. In an urban area in Senegal, mosquito exposure approximately halved with every 200 m increase in distance from a known mosquito breeding site (Trape *et al.* 1992). In a Malian village with one permanent breeding site and dense human inhabitation at 50-1000 m from this breeding site, there were evident hotspots of mosquito exposure in the dry season close to the main breeding site but mosquitoes were more dispersed during the wet season (Figure 1).

The pattern described in Figure 1 is typical for areas of seasonal malaria transmission, where mosquito exposure is commonly most clustered in the dry season when few permanent breeding sites exist (Lindsay *et al.* 1995, Trape *et al.* 1992). After seasonal rains the permanent breeding sites become more productive but alternative breeding sites also arise that are more widely distributed across the transmission focus, leading to a wider dispersal of mosquitoes. Seasonal mosquito

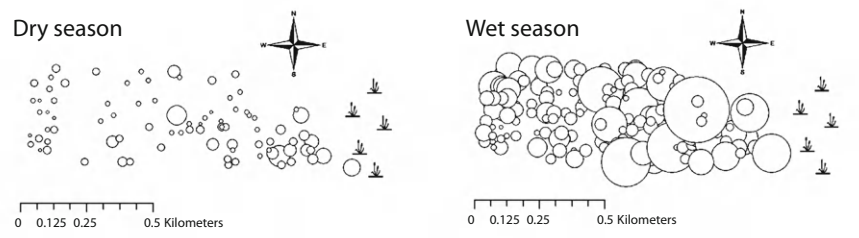

Figure 1. Mosquito catches in an area of moderate and seasonal malaria transmission in Mali. Mosquitoes were sampled in every household on a monthly basis. The size of the circles represents the average number of mosquitoes caught per households. Mosquito exposure was low and clustered near the main permanent breeding site in the dry season (left) but was higher and more widely dispersed during the wet season (right) (Bousema, unpublished observations).

dispersal patterns may also change under the influence of seasonal variations in wind patterns (Lindsay *et al.* 1995).

In areas where the human population is on average located further away from the breeding site, more sparsely distributed or where mosquitoes feed on human and non-human hosts, geographical patterns may be less distinct. In a rural area in Tanzania where households were located 800-2,000 m from a permanent river, where large numbers of cattle were present and *Anopheles arabiensis* Patton was the main vector, there was no clear association between mosquito exposure and distance to the river (Oesterholt *et al.* 2006).

Factors determining the contact rate between man and mosquito

In addition to the distance to mosquito breeding sites, elements that influence the contact rate between humans and mosquitoes have an obvious effect on the exposure to (infected) mosquitoes. The incidence of malaria and/or the exposure to mosquitoes has been associated with poorer housing conditions, especially incomplete housing (Gamage-Mendis *et al.* 1991), mud walls (Bousema *et al.* 2010a, Gamage-Mendis *et al.* 1991, Kirby *et al.* 2008), uneven wall structure (Bousema *et al.* 2010a), thatched roofs and ceiling (Bousema *et al.* 2010a, Gamage-Mendis *et al.* 1991, Lindsay *et al.* 1995, Ye *et al.* 2006), window screening (Oesterholt *et al.* 2006), window size (Oesterholt *et al.* 2006), presence of eaves (Kirby *et al.* 2008, Lindsay and Snow 1988, Lindsay *et al.* 1995), presence of animals (Kirby *et al.* 2008, Yamamoto *et al.* 2009) and the use of smoke or local incense to repel mosquitoes (Kirby *et al.* 2008, Lindsay *et al.* 1995, Yamamoto *et al.* 2009). Improvements in the house conditions, notably mosquito screening and closure of eaves, therefore lead to substantial reductions in mosquito exposure and can contribute to a reduction in anaemia in human inhabitants (Kirby *et al.* 2009). Other household protective measures such as indoor residual spraying (IRS) and insecticide treated nets (ITNs) have also been associated with reductions in malaria incidence (Bousema *et al.* 2010a, Bradley *et al.* 1986, Hawley *et al.* 2003, Pluess *et al.* 2010). In addition to a direct protective effect, reducing the risk of malaria in people sleeping under an ITN, there is evidence for a community effect of ITNs. In an area of intense malaria transmission in Western Kenya, it was described that ITNs exert a protective effect in compounds lacking ITNs that are located within 300 m of compounds with ITNs (Hawley *et al.*

2003). This indirect beneficial effect of ITNs is evident for child mortality, moderate anaemia and high-density parasitaemia (Hawley *et al.* 2003).

Regardless of the usage of protective measures, the attractiveness of humans also differs. Intra-individual mosquito attractiveness is related to variability in carbon dioxide (CO_2) release, ammonia, lactic acid, and other aliphatic carboxylic acids (Smallegange and Takken 2010). Pregnancy may increase attractiveness to mosquitoes by a factor 1.7-4.5 (Ansell *et al.* 2002), possibly as a result of differences in body surface, related to CO_2 release, temperature and odour. Non-pregnant individuals also differ in their attractiveness to mosquitoes and in their response to being bitten, resulting in differences in the number of bites experienced by individuals (Lindsay *et al.* 1993a). The intrinsic variation in attractiveness is probably largely mediated by sweat-associated human volatiles (Smallegange *et al.* 2011, Verhulst *et al.* 2009). This human odour profile has a genetic background (Roberts *et al.* 2005), explaining the genetic component that was previously associated with differential attractiveness (Kirk *et al.* 2000). Human leukocyte antigen (HLA) genes in particular may be involved in determining the intrinsic differential attractiveness of humans to mosquitoes (Verhulst *et al.* 2010) through their influence on the human body odour profile. The role of olfaction in vector-borne diseases is described in detail in a previous book in this series (Takken and Knols 2010).

Stability of hotspots of malaria transmission over time

To be of public health relevance, the geographical location of hotspots should be identifiable and show a certain consistency over time. If hotspots of malaria transmission change between seasons or years, it will become costly and logistically challenging to identify and target hotspots to have a beneficial impact on the burden of disease. The temporal stability and logistical identifiability are therefore crucial for utilizing hotspots of malaria transmission for effective malaria control. Some studies have reported that clusters of higher malaria incidence may change with time (Bejon *et al.* 2010, Coleman *et al.* 2009) while others indicated that they are remarkably stable over months or even years (Bautista *et al.* 2006, Bejon *et al.* 2010, Bousema *et al.* 2010a, Coleman *et al.* 2009, Ernst *et al.* 2006, Gaudart *et al.* 2006, Nourein *et al.* 2011). Unstable clusters of malaria incidence may reflect a problem in using malaria incidence for detecting hotspots, something that is explained in more detail in the Section 'Operational approaches to detecting hotspots of malaria transmission'. In areas of unstable malaria transmission, an unpredictable influx of malaria-infected individuals into an area with a suitable climate for malaria may also lead to different hotspots of malaria transmission over time (Coleman *et al.* 2009). Contrary to these important but relatively uncommon observations, most published reports indicate that hotspots of malaria transmission or mosquito exposure are stable over time. The most convincing evidence for this temporal stability of geographically defined hotspots comes from areas where transmission intensity decreased over time as a consequence of climatic changes or untargeted interventions but where hotspots of intense malaria transmission persisted (Bautista *et al.* 2006, Ernst *et al.* 2006, Gaudart *et al.* 2006, Nourein *et al.* 2011).

The most readily available data to describe the temporal consistency of hotspots of malaria transmission comes from entomological studies. Although we argued that the presence of mosquitoes does not automatically equal elevated malaria transmission intensity, field studies that sampled mosquitoes in the same households over several months provide valuable information on the consistency in exposure to malaria vectors. In Table 1, the findings from some of these studies from West and East Africa are summarised. Although the average number of mosquitoes differs tremendously between the wet and dry season, the same households are exposed to

Table 1.Consistency in mosquito exposure in three African settings.

	Ifakara, Tanzania (Akim *et al.* 2000, Drakeley *et al.* 2003)	Korogwe, Tanzania (Bousema *et al.* 2010a)	Sotuba, Mali
Location	Latitude 8° 8′ S Longitude 36° 41′ E	Latitude 5° 9′ S Longitude 38° 29′ E	Latitude 12° 40′ N Longitude 7° 55′ W
Parasite prevalence 2-9 year old children	55.1 (53.3-56.9)	28.6 (23.3-33.7)	6.1% (3.4-10.4)
Households sampled	32	185	254
Area sampled	4×5 km	5×6 km	0.8×0.7 km
Average *Anopheles* mosquito count wet season (range)	137.8 (4-1,266)	5.8 (0-129)	16.0 (0-184)
Average anopheles mosquito count early dry season/cool season (range)	1.4 (0-10.7)	1.3 (0-24)	7.9 (0-142)
Average *Anopheles* mosquito count end dry season/hot season (range)	0.4 (0-5.2)	0.50 (0-30)	1.2 (0-13)
Correlation household catches wet – early dry (*P*-value)	0.54 (0.0015)	0.30 (<0.001)	0.41 (<0.001)
Correlation household catches wet – end dry (*P*-value)	0.45 (0.01)	0.15 (0.05)	0.38 (<0.001)
Correlation household catches early – end dry (i-value)	0.72 (<0.001)	0.32 (<0.001)	0.42 (<0.001)

the highest relative number of mosquitoes. One could argue that this (statistically significant) consistency is unsurprising in relatively large geographical areas where environmental differences make certain sub-villages consistently more exposed to malaria than others (e.g. Korogwe and Ifakara in Table 1). However, the findings from households in a single village in Mali indicate that also at micro-epidemiological level, relative exposure to anophelines may be highly consistent over time despite temporal fluctuations in mosquito abundance.

Using hotspots for targeted malaria control

Do hotspots fuel wider malaria transmission?

There are several reasons to assume that hotspots form important reservoirs for further malaria transmission. In areas of very low and unstable malaria transmission, hotspots can be present throughout the seasons and form the only likely source of parasites for seasonal or epidemic increases in malaria transmission in the wider community (Ernst *et al.* 2006, Nourein *et al.* 2011). Mathematical models also consistently show that the overall level of transmission intensity is increased by heterogeneity in malaria transmission, suggesting a fuelling effect of hotspots of malaria transmission. In a seminal study by Woolhouse and colleagues, R_0 was 2-4 fold increased when heterogeneity in mosquito exposure was included in malaria transmission models (Woolhouse *et al.* 1997). It was later demonstrated that the impact of heterogeneity in mosquito exposure on transmission efficiency may differ between different settings (Smith *et al.* 2007): heterogeneity in mosquito exposure may augment malaria transmission in low endemic settings by allowing

mosquitoes to source their infections efficiently from hotspots; in areas of very high transmission intensity heterogeneity in mosquito exposure may result in segregation of populations where the clustering of mosquito exposure hinders instead of stimulates the spread of malaria. In more general terms, mosquitoes have to preferentially source their parasites from hotspots and subsequently spread their infection geographically to form a source of malaria transmission to a wider community. For this, the entire population of a focus of malaria transmission should be exposed to the same mosquito population while individuals living in hotspots should have more encounters with an otherwise randomly mixing mosquito population.

A study in Tanzania where mosquitoes were captured, marked with fluorescent powder, released and recaptured observed that 68% of mosquitoes returned to the same household as where they were initially captured (McCall *et al.* 2001) but these findings were not confirmed in follow-up experiments. In Papua New Guinea mosquitoes appeared to have a 'memorized' home range and limited dispersal range in the focus of malaria transmission they are accustomed to (Charlwood *et al.* 1988). These indications that mosquito populations may not mix randomly in villages need confirmation but would have important epidemiological consequences (Dye and Hasibeder 1986). In an extreme and highly unlikely scenario, no dispersal of mosquito populations from hotspots could result in intense transmission intensity inside hotspots of malaria transmission without consequences for the rest of the focus of malaria transmission that are exposed to a different mosquito population. A more likely scenario would be that there is a certain level of site fidelity and mosquitoes acquiring infections in hotspots transmit this infection to humans living outside hotspots but at a lower rate than is currently assumed by mathematical simulation models.

Will targeted interventions reduce malaria transmission?

Untargeted control efforts are relatively inefficient if heterogeneous transmission is assumed and high-risk households are missed (Carter *et al.* 2000, Dye and Hasibeder 1986). A disproportionate exclusion of hotspots of malaria transmission from malaria control measures is not a hypothetical scenario since several of the factors that have been associated with increased mosquito exposure are related to a lower socio-economic status which is in turn an important predictor of low participation in control methods. Population averaged coverage levels may therefore not accurately reflect the 'effective coverage' with malaria control measures.

There are several reasons why interventions targeted to hotspots of malaria transmission will be more efficient in reducing the burden of malaria than untargeted approaches. The most obvious is to protect individuals living in areas where the risk of (severe) malaria is highest. The largest, and most cost-efficient, impact on (severe) disease can be expected if those individuals who are most at risk preferentially receive protective measures. However, this does not reflect the full potential of hotspot targeted interventions. The additional and perhaps most attractive benefit of targeting hotspots of malaria transmission is that it can result in community-wide beneficial effects. By reducing or interrupting transmission in those households that contribute most to malaria transmission, community-wide malaria control may be improved in a cost-efficient manner. For this, a paradigm shift in thinking about malaria control is needed that focuses on epidemiologically relevant malaria cases instead of clinically vulnerable individuals (MalERA Consultative Group on Diagnoses and Diagnostics 2011). If the main objective of targeted interventions would be to protect individuals at risk of severe disease, an approach may be chosen where the known risk groups of (severe) disease are preferentially included in interventions. Although this will reduce morbidity and mortality in clinically relevant risk groups, the public health impact may be limited because other parts of the population can be equally important for disease transmission

(Bousema and Drakeley 2011, Drakeley *et al.* 2000). Asymptomatic individuals of all age groups play an important role in maintaining malaria transmission (Okell *et al.* 2008, Ouedraogo *et al.* 2009) and are particularly common in hotspots of malaria transmission (Bejon *et al.* 2010, Ernst *et al.* 2006, Stresman *et al.* 2010). To maximize the impact of targeted interventions, these interventions should therefore aim to eliminate malaria transmission in and from the hotspot. For this, a comprehensive approach is needed where conventional vector control methods such as ITNs and IRS can be combined with more laborious but efficacious vector control tools such as larviciding (Fillinger *et al.* 2009a) and interventions that aim to reduce the human infectious reservoir of malaria. Interventions that aim to clear the human infectious reservoir of malaria may include tools that are currently deemed less suitable for community-wide coverage such as mass drug administration with antimalarial drugs (Okell *et al.* 2011), focal screening of asymptomatic individuals followed by antimalarial treatment (Okell *et al.* 2011, Stresman *et al.* 2010) and employment of (transmission-blocking) malaria vaccines in all age groups (MalERA Consultative Group on Vaccines 2011, Sauerwein 2007).

Mathematical simulations suggest that perfectly targeted malaria control efforts can have an impact that is up to 4-fold higher than that of untargeted control efforts (Carter *et al.* 2000, Smith *et al.* 2007). As discussed in the previous paragraph, these estimates are influenced by epidemiological characteristics of the transmission setting (notably spatial patterns in population density and mixing patterns in mosquito populations (Dye and Hasibeder 1986, Smith *et al.* 2007)) and may need to be adjusted in the context of mosquito site fidelity. Nevertheless, all current evidence suggests a beneficial effect of targeted control efforts if (1) hotspots of malaria transmission can be operationally identified; (2) this information allows logistically feasible targeting; (3) the benefit of a higher efficiency of interventions financially outweighs the costs of detecting hotspots of malaria transmission (Carter *et al.* 2000). The first hurdle to take is to define an operational approach for detecting hotspots of malaria transmission.

Operational approaches to detecting hotspots of malaria transmission

Hotspots of malaria transmission have been detected based on variations in the human and in the vector components of malaria transmission. Micro-epidemiological elevations in malaria incidence are often used as evidence for malaria hotspots (Bejon *et al.* 2010, 2011a, Bousema *et al.* 2010a, Brooker *et al.* 2004, Clark *et al.* 2008). In addition, elevations in (asymptomatic) parasite prevalence (Bejon *et al.* 2010, Cook *et al.* 2011, Pullan *et al.* 2011), serological responses to malaria-specific antigens (Bousema *et al.* 2010a,c, Cook *et al.* 2011), mosquito abundance (Bousema *et al.* 2010a) and exposure to infected mosquitoes (Bousema *et al.* 2010a) have been utilized in attempts to quantify micro-epidemiological variations in malaria risk.

Entomological indicators

The most direct evidence of a hotspot of malaria transmission would be an increased exposure to infected mosquito bites. This gold standard measure for defining transmission intensity is difficult to assess at micro-epidemiological level: it depends on intensive sampling of mosquitoes over time and space and the detection of parasite stages in the mosquito salivary glands by microscopical examination or enzyme-linked immunosorbent assay (ELISA) (Wirtz *et al.* 1987). This makes entomological evaluations very laborious, especially in areas with low vector densities where currently available mosquito sampling tools lose sensitivity (Hamad *et al.* 2002, Oesterholt *et al.* 2006). An additional problem is the current uncertainty about the best mosquito sampling tool. Repeated sampling over time and at multiple locations require simple and affordable tools

such as miniature light traps, odour baited traps or pyrethrum spray catches. Water storage clay pot traps were piloted as low-cost affordable approach that can be used for large-scale sampling (Odiere *et al.* 2007) but have serious limitations in reliably sampling mosquitoes in field settings (Van den Bijllaardt *et al.* 2009). Importantly, sampling strategies for outdoor biting and resting mosquitoes are poorly standardized. This creates a risk of ignoring important vector populations that have a preference for outdoor biting and resting (Riehle *et al.* 2011). These limitations make it operationally unattractive to depend on entomological assessments of malaria exposure to define hotspots of malaria transmission in most endemic settings.

Human indicators

More indirect but more easily accessible evidence for hotspots of malaria transmission may come from detecting (the consequences of) malaria infections in humans. The overarching advantage of relying on infections in humans is that this circumvents any problems in low densities or indoor/outdoor biting mosquitoes. Heterogeneity in malaria incidence is a frequently used indicator of increased exposure but its validity is affected by the differential acquisition of immunity inside and outside hotspots and treatment seeking behaviour. In areas that are consistently exposed to higher levels of malaria transmission, immunity may be acquired at a faster rate and as a consequence fewer infections result in clinical malaria. Clinical malaria and high density parasitaemia may therefore be lowest in areas exposed to higher levels of transmission intensity where people have acquired protective immunity more rapidly (Clarke *et al.* 2002, Thomas and Lindsay 2000). Clinical incidence may therefore give inaccurate estimates if measured in age-groups where residence in a hotspot of malaria transmission has resulted in an effective immune response.

This confounding effect of immunity may be less prominent for asymptomatic parasite carriage. Asymptomatic parasite carriage may last several months (Falk *et al.* 2006) making estimates more robust in settings where the clinical infrastructure allows rapid treatment of symptomatic infections. Most importantly, immune responses that prevent infection are acquired later in life compared to clinical immunity (Smith *et al.* 2005); low density infections in hyper immune adults suggest that immunity effectively preventing malaria infection may actually be very rare (Okell *et al.* 2009, Proietti *et al.* 2011). This suggests that clustering of asexual parasite carriage may form a more stable indicator of transmission intensity than clinical malaria episodes (Bejon *et al.* 2010).

A third option to utilize malaria infections in humans as indirect indicator of higher malaria exposure is formed by serological markers of malaria exposure. Antibody responses to malaria specific antigens are acquired in response to (cumulative) exposure and can be used to define small-scale variations in malaria exposure (Bousema *et al.* 2010a,b, Drakeley *et al.* 2005). Because antibody responses are relatively long-lived, serological markers of malaria exposure are likely to be most suitable for detecting stable hotspots of malaria transmission (Bousema *et al.* 2010a) and most sensitive in areas of low endemicity (Corran *et al.* 2007). The strong age-dependency of antibody responses necessitates an analysis of an age-dependent conversion rate from sero-negative to sero-positive (Bousema *et al.* 2010a, Drakeley *et al.* 2005) or an age-adjusted antibody density (Bousema *et al.* 2010c, Wilson *et al.* 2007). Human genetic polymorphisms that modulate the risk of clinical and asymptomatic parasite carriage (see above) may also influence malaria specific antibody responses (Sarr *et al.* 2006). The importance of this immune modulating effect remains to be established but could alter the sensitivity of serological markers of exposure in identifying hotspots of malaria transmission in areas where human genetic polymorphisms show spatial heterogeneity.

Spatial analysis on entomological or human indicators

The level of statistical significance is a relevant factor in determining whether a certain geographical area forms a plausible hotspot of malaria transmission. A powerful statistical tool that is frequently used to analyse spatial and spatio-temporal patterns is SaTScan (SatScan). The SaTScan software is freely available online and uses a Kulldorf spatial scan statistic (Coleman *et al.* 2009) to detect clusters in space and (if requested) time. A scanning window is used that moves across space and counts the observed and expected number of cases or attributes of cases for each location and size of the window. For the sake of simplicity, the remainder of the text will assume a case-control approach (Bernouilli model) although SaTScan also allows scans on continuous or categorical variables and allows the detection of hotspots (higher count than expected) as well as coldspots (lower count than expected). In the case-control approach, the window with the greatest ratio of observed to expected cases is noted. The statistical significance of this hotspot or coldspot is then evaluated taking into account the multiple tests for the many potential cluster locations and sizes evaluated. The output of the SaTScan analysis gives the location, size and level of statistical significance of the most likely clusters.

One important characteristic of the SaTScan approach is that it calculates the number of expected cases by considering an even distribution of cases across the population and is very susceptible to the restrictions given by the user (e.g. maximum window size, allowance of overlapping clusters). This means that a spatial scan on a large geographical area will only pick up the most extreme hotspots. An additional complication with spatial scans on patterns in the occurrence of malaria is formed by the strong seasonal fluctuations in malaria incidence. Spatial scans on variables that are less susceptible to these seasonal fluctuations such as cumulative malaria incidence over several seasons (Bejon *et al.* 2010, Bousema *et al.* 2010a), cumulative parasite prevalence (Bejon *et al.* 2010) or long-lived antibodies to malaria antigens (Bejon *et al.* 2010, 2011b, Bejon *et al.* 2010, Bousema *et al.* 2010a,b) are likely to produce most robust results. The scans can be performed separately for individual villages to increase the likelihood of detecting hotspots that are of local relevance but that would not have been detected in an area-wide scan.

Environmental models

Environmental factors have long been associated with individual malaria risk. The simplest environmental models for detecting spatial variation in malaria risk incorporate distance to plausible mosquito breeding sites. These models have some value in predicting malaria risk (Carter *et al.* 2000, Clark *et al.* 2008, Kreuels *et al.* 2008, Oesterholt *et al.* 2006) but failed to explain hotspots of malaria transmission in two recent studies (Bejon *et al.* 2010, Bousema *et al.* 2010a). In reality, the prediction of hotspots of malaria transmission is relatively straightforward in foci of malaria transmission with a single or very limited number of plausible sources of mosquito emergence are present. In other settings the correlation between malaria risk and distance to water may be weak (Bousema *et al.* 2010a) and additional factors have to be incorporated in models to reach a sensitivity that justifies rationally targeting malaria control. In these circumstances site-specific models may have to be prepared to encapsulate all locally relevant predictors of malaria risk. This makes environmental models less attractive from a public health perspective.

A more sophisticated form of environmental modelling that may partly overcome the necessity for on-site data-collection to define local malaria risk factors is an approach that utilizes remote sensing data to determine factors such as elevation, daytime and night-time temperature, humidity, vegetation, soil moisture content, etc. The Malaria Atlas Project incorporates

epidemiological data on parasite prevalence, environmental covariates and human settlement data and has resulted in malaria risk maps for different endemic regions (Hay *et al.* 2009, 2010, Malaria Atlas Project undated). A current limitation for utilizing environmental data for detection hotspots of malaria transmission is that is routinely available remote sensing data has limited spatial resolution, typically 8 km^2 or 1 km^2 per pixel (Bejon *et al.* 2010, Hay *et al.* 2006). Some data is available at a higher resolution but have been validated less widely and currently do not benefit from processing by a temporal algorithm (Fourier), an approach that normalizes readings while preserving the seasonal variation in measures (Hay *et al.* 2006). As a consequence, environmental models are currently not validated for detecting hotspots of malaria transmission with malaria transmission foci.

Operational approaches to target hotspots of malaria transmission

Once hotspots of malaria transmission are detected, they can be targeted with conventional and less conventional malaria control tools. Hotspot-targeted interventions are likely to be beneficial for people living inside and people living outside the targeted hotspots (see Section 'Will targeted interventions reduce malaria transmission?'). The most straightforward approach of targeting hotspots is formed by the local up-scaling of efficacious conventional vector control tools such as ITNs and IRS. However, this approach will only target indoor biting and/or resting vectors while there is accumulating evidence that outdoor biting vectors are becoming increasingly important for malaria transmission (Reddy *et al.* 2011, Russell *et al.* 2011). The very essence of hotspot targeted interventions, focal interventions to protect the community at large, allows more laborious interventions that become operationally attractive because these require implementation in a fraction of the malaria endemic area only. Vector control tools that form attractive components of intensive hotspot-targeted interventions include larviciding of mosquito breeding sites (Fillinger *et al.* 2008) and the use of entomopathogenic fungi (Knols *et al.* 2010) for the control of adult mosquitoes. Both tools require frequent re-application that makes them less attractive for community-wide interventions but this shortcoming does not necessarily hinder targeted implementations. Similarly, the use of odour-baited mosquito traps (Jawara *et al.* 2011, Okumu *et al.* 2010) as part of community-wide interventions is currently unattractive but a targeted push-pull approach where mosquitoes are deterred from households in hotspots with a repellent and lured into traps with synthetic human odours may hold promise.

Intensive vector control in hotspots can be supported by interventions that aim to reduce the transmission potential of the human host. The human infectious reservoir can be reduced by improving malaria case management in hotspots, thereby reducing the duration of parasite and gametocyte carriage in clinical malaria cases (Bousema *et al.* 2010b). Because a large proportion of parasite carriers in hotspots may harbour their parasites without experiencing symptoms (Stresman *et al.* 2010), and therefore without actively seeking treatment, a more aggressive approach to clear the human infectious reservoir will be beneficial to reduce malaria transmission. The most inclusive approach is formed by mass treatment campaigns where all individuals, regardless of symptoms, receive a full therapeutic dose of antimalarials (Von Seidlein and Greenwood 2003). Apart from ethical issues with mass drug administration, an operational drawback is that it may require several rounds to be efficacious (Okell *et al.* 2011). This makes deployment of mass treatment campaigns most attractive in the form of targeted interventions. Mass drug administrations are laborious and ethically challenging in asymptomatic individuals who do not experience a personal benefit from treatment. In targeted interventions, the additional efforts to clear the human infectious reservoir are justified by the increased importance of the targeted parasite carriers for overall malaria transmission.

A last operational element that is important in identifying and targeting hotspots of malaria transmission concerns human movement patterns. Parasitaemic individuals may contribute to hotspots of malaria transmission in low endemic settings, as was suggested for the Kenyan highlands (Ernst *et al.* 2006). In addition, the effect of interventions targeted to the human population (e.g. treatment campaigns) may be diminished by the migration of parasitaemic and untargeted individuals. A comprehensive approach where parasites are targeted in humans and mosquitoes is likely to be less affected by human movement patterns.

Conclusions

- Spatial variation in malaria incidence and exposure to malaria-infected mosquitoes is present at all levels of transmission intensity.
- Hotspots of malaria transmission are geographical parts of foci of malaria transmission that are characterised by an increased level of transmission intensity compared to the average value of the focus.
- Human genetic factors contribute considerably to individual variation in malaria risk but with exception of areas where genetic traits cluster at micro-epidemiological level, do not explain hotspots of malaria transmission.
- There is some evidence that human genetic factors contribute specifically to transmission stages of malaria parasites and their infectivity to mosquitoes. These findings require confirmation in future studies but could be of epidemiological relevance in explaining malaria transmission patterns and planning malaria control efforts.
- Spatial variation in mosquito exposure is remarkably consistent despite large seasonal fluctuations in mosquito densities. Within regions and individual villages, the same households can be exposed to consistently higher mosquito numbers.
- The spread of malaria infections from hotspots to the wider community is biologically plausible but needs prospective confirmation and quantification to support the planning of hotspot-targeted interventions.
- Hotspot-targeted interventions should aim at reducing or preventing malaria transmission in and from the hotspot, not at protecting vulnerable individuals living in a hotspot of malaria transmission.
- Hotspots of malaria transmission can be identified by entomological and human parameters. Malaria incidence is an unreliable indicator of hotspots of malaria transmission unless restricted to age-groups that have limited clinical immunity; malaria parasite carriage or the presence or density of serological markers of malaria exposure may be more robust indicators of heterogeneity in malaria exposure.

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12. Considerations for male fitness in successful genetic vector control programs

Michelle E.H. Helinski and Laura C. Harrington

Abstract

A number of genetic control strategies, including sterile, transgenic, and *Wolbachia*-based approaches, are under development to reduce mosquito vector populations and the impact of important diseases such as malaria and dengue. Fitness of released males is critically important for the success of these strategies. In order to understand how to optimise the success of released males, we need to determine how males behave in their natural environment and which factors contribute to their success. Male mosquito biology has received little attention, although recent contributions have advanced our knowledge in this area. These advances include the discovery of precopulatory acoustic interactions and the identification of seminal fluid proteins that may have profound effects on female physiology and behaviour. Gaps in our knowledge include detailed information on male survival, dispersal, and mating strategies in the field across a range of geographical and ecological settings in which genetic control strategies may be deployed. There is evidence from laboratory studies that age, body size, and conditions during larval development contribute to male mating success; however, verification of these findings in natural settings is lacking. Although fitness assessments of transgenic or sterile males in genetic control programs are often performed in the laboratory using laboratory-adapted colonies, we advocate that fitness studies are conducted in semi-field cages under ambient conditions where insects are challenged with wild-type mosquitoes to determine their true potential. Because of the considerable amount of time and costs involved with the execution of cage trials we recommend that researchers plan field cage studies as early as possible in the assessment process to prevent time delays.

Keywords: genetic control, mosquitoes, male fitness, reproductive success, field applications

Introduction

The potential to control mosquitoes and vector-borne diseases with genetic methods (genetic vector control) has captivated the interest of scientists since the 1960s (Craig 1970, Knipling 1955), despite setbacks with early efforts. Existing approaches for controlling vector-borne diseases all face major challenges such as parasite and vector resistance, lack of funding, high cost of insecticide applications, and lack of local infrastructure to carry out control programs. Thus, new control strategies are critically needed. Genetic vector control has a number of advantages that make it a suitable strategy to be explored for mosquitoes. It is an environmentally friendly method that, unlike the use of many insecticides, is species-specific. Furthermore, it has the potential to impact populations of mosquito species which may not be reached by conventional control methods such as indoor residual spraying and bednets (ITN). These include species that tend to rest and feed outdoors including some *Anopheles gambiae* Giles and *Anopheles arabiensis* Patton (Fornadel *et al.* 2010, Riehle *et al.* 2011), species that have recently shifted to exophagy in response to ITN selection pressure (Russell *et al.* 2011), and *An. arabiensis* populations with shifts to earlier peak biting times to contact hosts before they retire under their bed nets (Yohannes and Boelee 2012). Another benefit of genetic vector control is the potential for minimal costs to residents in treatment areas and lower long term costs for vector control by organised government or private control programs.

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Despite the benefits of genetic vector control, numerous downsides exist as illustrated by some historical failures. Prior attempts were unsuccessful due to altered mosquito biology and behaviour through laboratory adaptation, lack of trust and cooperation by local community residents and governments where control was deployed, and numerous unknown factors leading to reduced fitness of the modified mosquito strains. The latter issue has been a major impetus for greater research on basic aspects of mosquito biology and behaviour. Additionally, in areas where a multitude of vectors exist, the feasibility of applying a separate genetic control strategy against each vector species is doubtful.

There are a number of obstacles and gaps in our understanding of mosquito biology that need to be addressed if genetic vector control will be successful. One of these key areas is male biology. Most genetic control strategies under consideration today utilise releases of males for ethical and safety reasons. In this chapter, we focus our review on male biology and fitness. We begin with a brief overview of the various genetic control strategies that have been developed against mosquitoes. In the second half of the chapter, we address what is known about male mosquito mating biology and the knowledge gleaned to date on male reproductive success. In addition, we will present some recent advances demonstrating that the mating system of mosquitoes is more complex than previously assumed. Next, we summarise the studies of transgenic and sterile insect fitness conducted to date and provide a discussion of the biological challenges released males face to be reproductively successful. Finally, we highlight key aspects of mosquito biology that should be explored and discuss performance indicators that can be used to assess strain fitness.

Overview existing genetic control strategies

Classic genetic control

Perhaps the most well-known genetic control strategy is the sterile insect technique (SIT). This approach involves releasing large numbers of sterilised males into the wild population (Knipling 1955), with sterility being achieved by chemosterilization or ionising radiation. With this strategy, sterile males are intended to mate with wild-type females, resulting in no offspring and ultimately leading to population reduction. If repeated, inundative releases (i.e. the release of overwhelming numbers of sterile males) are deployed with SIT, population elimination may be achieved. SIT has been used successfully in the eradication of other Dipterans such as the New World Screwworm fly *Cochliomyia hominivorax* from the USA and Central America (Snow 1988), as well as on-going population reduction of the Mediterranean fruit fly *Ceratitis capitata* from South and Central America (Dyck *et al.* 2005).

A number of field releases have been performed to test the potential of genetic control strategies for mosquitoes (see Benedict and Robinson (2003) and Asman *et al.* (1981) for a detailed overview). The release of chemosterilised *Anopheles albimanus* Wiedemman males in El Salvador was successful in reducing the native population (Lofgren *et al.* 1974). In addition, the release of chemosterilised male *Culex pipiens quinquefasciatus* Say successfully reduced a wild-type island population in Florida (Patterson *et al.* 1970), and chemosterilised *Aedes aegypti* L. males were competitive against low generation wild-type males when field-tested in Florida (Seawright *et al.* 1977). Other field releases were less successful. For example, the release of millions of irradiated male *Ae. aegypti* in Florida did not result in a noticeable population reduction (Morlan *et al.* 1962), nor did the release of irradiated *Anopheles quadrimaculatus* Say males in Florida (Weidhaas *et al.* 1962). Failures were attributed to behavioural differences between the release and wild-type strain, a lack of fitness of released males, and immigration of mated females from neighbouring

non-release sites, among other reasons (Benedict and Robinson 2003). One of the most notorious field trials for genetic mosquito control was conducted in India where accusations of biological warfare by the media and politicians led to complete discontinuation of SIT control efforts for decades (Curtis 2006). The experience in India highlighted the critical importance of carefully informing, ensuring consent and working in partnership with community residents and other stakeholders prior to a release.

Germ-line transformations strategies

Germ-line transformation strategies have received considerable interest for mosquito control in the last two decades (Alphey 2002, Alphey *et al.* 2002, Benedict and Robinson 2003, Catteruccia *et al.* 2005, Coates *et al.* 1998, Scott *et al.* 2002, Thomas *et al.* 2000). Germ-line transformation relies on the insertion of an alien segment of DNA into the genome (transgenesis) using embryonic microinjection to create a genetically modified mosquito (GMM). A number of mosquito species have been successfully transformed. GMM strategies for vector/disease control can be divided into two approaches: (1) population replacement strategies where engineered strains that carry pathogen resistant genes are released to spread the transgene into the susceptible populations, and (2) population reduction strategies where engineered strains are released to reduce or eliminate the local wild-type population in a manner similar to SIT releases.

Pathogen-refractory strains

Genetic transformation can be used to engineer strains of mosquitoes that are refractory to pathogens, and a number of such strains have been produced to date. Transgenic *Anopheles stephensi* Liston were developed that expressed a bee venom (Moreira *et al.* 2002) or an anti-parasitic gene (Ito *et al.* 2002) which resulted in a reduction of *Plasmodium berghei* Vincke and Zips parasites. A few other strains were created with a similar approach. For example, *Aedes fluviatilis* Lutz was created that expressed mutated bee venom protein against *Plasmodium gallinaceum* Brumpt (Rodrigues *et al.* 2008), and *An. gambiae* expressed antimicrobial peptide cecropin A against *P. berghei* (Kim *et al.* 2004). In both lines reduced oocyst loads after infectious blood meals were observed. In addition, a strain of *An. gambiae* was engineered that expressed the synthetic anti-malaria gene Vida3 which reduced *Plasmodium yoelii nigeriensis* density by 85% (Meredith *et al.* 2011). Although the development of such strains is encouraging, they all act against non-human malarias (rodent or bird). Recently, however, a strain of *An. stephensi* was engineered with increased expression of an important protein (Akt) in immune defence (Corby-Harris *et al.* 2010). This strain had high levels of resistance to the main human malaria parasite *P. falciparum*. Carter and Hurd (2010) identified a number of antimicrobial peptides that act against *Plasmodium*. Discovery of these promising candidates for integration into the mosquito genome should advance the field. Besides efforts to target malaria parasites, a strain of *Ae. aegypti* (Carb77) was engineered with a virus-derived, inverted repeat that triggered RNA interference in the midgut to control dengue virus 2 (DEN 2) (Franz *et al.* 2006). However, Franz *et al.* (2009) recently reported that this line lost the ability to silence the DEN 2 genome due to a loss of effector gene expression.

Lethal and sexing systems

Transgenesis can also be used to generate a sterilising system based on dominant lethal genes expressed either in both sexes or in females alone. This approach is called RIDL for 'release of insects carrying a dominant lethal' (Alphey 2002). Recently, a strain was constructed in *Ae. aegypti* that exhibits a late-acting, female-specific lethality (Fu *et al.* 2010), which can be used for

population reduction strategies. Other potentially suitable candidates that could cause offspring lethality, such as cytotoxic proteins or proapoptotic gene products, are discussed in Catteruccia *et al.* (2009) and Nolan *et al.* (2011).

Transgenic insects are usually engineered to express a reporter gene for a fluorescent protein. This marking can be used to facilitate sex separation of adults, which is an essential prerequisite for any genetic control program relying on release of one sex only (Robinson and Franz 2000). A transgenic sexing strain was developed in *An. stephensi* in which only male mosquitoes expressed a fluorescent protein in their gonads, and males could be identified by automated screening (Catteruccia *et al.* 2005). The RIDL strain discussed above can also permit removal of females due to the tetracycline repressible sex-specific flightless phenotype (Fu *et al.* 2010). As a consequence females die before eclosion or are unable to fly and function as adults and can be easily removed from the population.

Paratransgenesis

In addition to mosquito transformation, mosquito endosymbionts can be targeted for transgenesis. In paratransgenesis, genetically modified bacteria or viruses are used to deliver an effector molecule that inhibits the disease-causing pathogen in the insect gut (Beard *et al.* 2002). This technology has shown promise in controlling transmission of Chagas disease by Reduviid bugs (Beard *et al.* 2001), and is under consideration for tsetse flies (Aksoy *et al.* 2008). Densoviruses are a class of viruses that may be used to express molecules targeting pathogen development in mosquitoes, and can infect *Ae. aegypti* (Ward *et al.* 2001) and *An. gambiae* (Ren *et al.* 2008). In addition, bacteria of the genus *Asaia* are stably associated with *An. stephensi* (Favia *et al.* 2007), and appear to be good candidates for paratransgenesis (Aksoy 2008).

Wolbachia and other control strategies

Wolbachia bacteria provide another promising strategy to control mosquitoes. These bacteria can act as a gene-drive system for population replacement strategies, or can be used to spread cytoplasmic incompatibility (CI) (Dobson *et al.* 2002, Sinkins and O'Neill 2000). The use of *Wolbachia* to induce cytoplasmic incompatibility (CI) and suppress wild populations has been successfully demonstrated in *Cx. pipiens* in the 1960s (Laven 1967). A technique to transfer *Wolbachia* strains to develop new CI crossing types was developed in *Aedes albopictus* Skuse (Xi *et al.* 2005a), and this strain was successfully transfected with the *w*Ri strain of *Drosophila simulans* Sturtevant resulting in CI with naturally infected mosquitoes (Xi *et al.* 2006). In addition, the *Wolbachia* strain of *Aedes riversi* Bohart and Ingram has been introgressed into *Aedes polynesiensis* Marks resulting in CI (Brelsfoard *et al.* 2008). Other strains of *Wolbachia* have been explored; the introduction of the life-shortening *Wolbachia* strain *w*MelPop in *Ae. aegypti* resulted in a 50% reduction of adult life span under laboratory conditions (McMeniman *et al.* 2009). Since only older females have surpassed the pathogen/parasite intrinsic incubation period in order to infect blood hosts, the introduction of a life shortening strain could reduce disease transmission. The same *Wolbachia* infection also limited susceptibility of *Ae. aegypti* to dengue, Chikungunya and *Plasmodium* pathogens (Moreira *et al.* 2009). Introduction of *w*MelPop in *An. gambiae* resulted in a reduction of *Plasmodium* infection intensity due to the up-regulation of immune genes (Kambris *et al.* 2010). Recently, the *Wolbachia* strain *w*Mel was introduced into *Ae. aegypti* and this strain, as well as *w*MelPop, strongly inhibited dengue-2 virus replication, thereby blocking transmission (Walker *et al.* 2011). The *w*Mel strain was successful in invading a semi-field population of *Ae. aegypti* within a few generations after introduction (Walker *et al.* 2011).

Genetic transformation of entomopathogenic fungi presents another avenue to control mosquitoes using transgenesis. Recently, the entomopathogenic fungus *Metarhizium anisopliae* was transformed to express anti-sporozoite molecules, targeting that life stage in mosquitoes (Fang *et al.* 2011). This fungus infects a wide range of important malaria mosquito vectors, and, as a consequence, could be a new tool to reduce disease. However, the viability of the transgenic spores needs to be assessed to determine the cost-effectiveness of this method (Koenraadt and Takken 2011).

Potential application and limitations

Of all the approaches discussed above, the RIDL strategy is arguably furthest along at the time of writing with a months-long open field release trial performed in Grand Cayman in 2010 (Enserink 2010, Harris *et al.* 2011), a one-time open field release in Malaysia in 2011 (Enserink 2011), and field releases in Brazil in 2011 (Oxitec 2011). Another promising approach is with the *w*Mel strain of *Wolbachia* infected *Ae. aegypti*. Hoffmann *et al.* (2011) released these mosquitoes for a number of weeks in two open field sites in Australia, and *Wolbachia* successfully invaded the local populations. The next step will be to test the ability of this strain to reduce dengue transmission in an endemic setting. In addition to the RIDL and *w*Mel strains, a *Wolbachia* induced CI strategy is being evaluated to control *Ae. polynesiensis* in French Polynesia (Chambers *et al.* 2011).

There are a number of limitations to germ-line transformation approaches for mosquito control. For example, a suitable driving mechanism to deliver transgenes or recombinant bacteria through wild populations is essential for population replacement strategies. Transposable elements, *Wolbachia*, and meiotic drive have been proposed among other strategies but all face major limitations (James 2005, Sinkins and Gould 2006, Xi *et al.* 2005b). A drive system relying on a selfish genetic element known as Medea has been successfully demonstrated in *Drosophila* (Chen *et al.* 2007) but this technology has not been transferred to mosquitoes (Hay *et al.* 2010). Homing endonuclease-based gene drive systems are a novel method to drive transgenes through a population, and recent work in *An. gambiae* demonstrated the feasibility of this approach (Windbichler *et al.* 2011). In addition to technical issues, the release of transgenic insects requires pre-establishment of a significant social, political and ethical framework. These topics fall outside the scope of this chapter but nevertheless are of vital importance for the success of any genetic control program. For more information regarding these issues see Knols *et al.* (2007), Touré and Manga (2000), and Lavery *et al.* (2008).

Mosquito biology

Genetic control strategies depend on the ability of released males to successfully mate with females in the local population. Few aspects of mosquito mating biology are known because male mating biology has received little attention (Ferguson *et al.* 2005, 2010), although more studies have been recently undertaken in this area (see for example (Helinski and Harrington 2011, Huho *et al.* 2007, Ng'habi *et al.* 2005, Ponlawat and Harrington 2009). There are a variety of reasons for minimal progress in understanding male mating biology including lack of support by major funding agencies, lack of recognition of its importance by the vector biology community, and a lack of training for vector biologists in the area of mating biology and insect behaviour. Below, we provide a general introduction to mosquito mating with emphasis on the *Anopheles* and *Aedes* genera; for more extensive reviews of the mating system of mosquitoes see Yuval (2006) or Clements (1999). Then, we discuss the components contributing to male reproductive success.

Mating strategies

Most mosquitoes in the *Anopheles* genus tend to mate in crepuscular swarms (Charlwood *et al.* 2002b, Charlwood *et al.* 2003, Marchand 1984, Yuval 2006). Swarms are often formed over landmarks described as 'swarm markers' (Takken and Knols 1999). These aggregations can reach considerable size and are primarily composed of males (see Howell and Knols (2009) for a review). Females entering swarms are recognised by their wing beat frequency, and are actively pursued by males. Copulation usually occurs in flight, and lasts approximately between 8-40 seconds. Recent advances in stereoscopic video imaging techniques allow for a more in depth analysis of mating swarms, such as the location of individual males in a swarm (Manoukis *et al.* 2009), and swarm size and mating success between swarms (Diabate *et al.* 2011). With the help of these technologies, it might be possible to investigate certain male features (e.g. location in swarm, timing of swarm entry, flight patterns) that may increase the chance of obtaining a mate, and how females behave once entering a swarm. Studies on the swarming behaviour of the M and S molecular forms of *An. gambiae* in West-Africa provided evidence that spatial segregation of swarms contributed to reproductive isolation between forms (Diabate *et al.* 2009). However, the underlying mechanism for swarm site selection by males and how swarms are sustained remains unknown. Aggregation pheromones may play a role (Clements 1999), but few studies support this to date (Cabrera and Jaffe 2007). Contact pheromones were postulated to play a role in mosquito interactions (Nijhout and Craig 1971); one study observed a change in cuticular hydrocarbons after mating in *Ae. aegypti* and *An. gambiae* (Polerstock *et al.* 2002), and a role for species or molecular form recognition was suggested for field-collected *An. gambiae* and *An. arabiensis* (Caputo *et al.* 2007). However, no direct evidence has been found to date to support the presence of contact pheromones or the existence of form recognition in swarms (Diabate *et al.* 2009).

Swarming is costly for males as energetic reserves are depleted (Yuval *et al.* 1994, Yuval *et al.* 1993) and swarms can be susceptible to heavy predation (Yuval and Bouskila 1993). In many endemic settings, mating swarms have been observed in some areas but are hard to find in others (Takken and Knols 1999). This observation suggests that alternative mating strategies may be employed with the anophelines and some indirect evidence has demonstrated indoor mating in *An. gambiae* (Dao *et al.* 2008). Besides anophelines, members of other mosquito genera also swarm outdoors (e.g. *Cx. quinquefasciatus* (Gibson 1985)).

While many mosquitoes swarm outdoors and do not respond to host cues, *Ae. aegypti* and *Ae. albopictus* are frequently observed swarming in close proximity to the host (Cator *et al.* 2011, Gubler and Bhattacharya 1972, Hartberg 1971, Yuval 2006). Observations of the mating behaviour of *Ae. albopictus* around human hosts were performed outdoors in India (Gubler and Bhattacharya 1972), and outdoors for *Ae. aegypti* in Tanzania (Hartberg 1971). Observations were made in the early morning (i.e. between 5.30-9.00 AM for *Ae. aegypti*, and within 2 h after sunrise for *Ae. albopictus*). *Ae. albopictus* swarms formed around the observer's feet and ankles, while *Ae. aegypti* swarmed around the legs and head. *Ae. albopictus* swarms varied considerably in size from a few individuals up to 30-40 males (Gubler and Bhattacharya 1972). Swarming activity was high for the first 15 min, and then declined. Females were observed after a couple of minutes after swarm formation (Gubler and Bhattacharya 1972). In contrast, *Ae. aegypti* females arrived at the same time as the males (Hartberg 1971). Copulation occurred in flight in both species, and lasted 5-10 sec on average for *Ae. albopictus.* Swarms of *Ae. albopictus* were also observed over non-host markers such as trees (Gubler and Bhattacharya 1972). The presence of *Ae. albopictus* swarms over non-host markers was also reported in a study in Italy, however no details on the location and composition of the swarms were provided (Bellini *et al.* 2010). These accounts do suggest

the possibility that *Ae. albopictus* do not swarm exclusively around the host and this should be examined in more detail. In general, more extensive research should be performed to observe mating behaviour for both species in field settings across their geographical distribution range.

Acoustic interactions and mate choice

Recent advances in acoustic interactions in *Aedes* and *Anopheles* have reversed the textbook wisdom that female mosquitoes could not hear or attend to male flight tones. These studies indicate that males and females hear each other within 2-3 body lengths and modify their harmonic flight tone frequencies prior to mating such that they converge (Cator *et al.* 2009, Pennetier *et al.* 2010, Warren *et al.* 2009). In many mating systems, pre-mating acoustic interactions provide animals with information about the quality of a potential mate (Searcy and Nowicki 2005). This discovery in both *Ae. aegypti* and *An. gambiae* suggests the potential for flight tone as a mate assessment tool (Cator *et al.* 2010). A study with *Ae. aegypti* demonstrated that acoustic convergence behaviour is related to higher mating success and is heritable (Cator and Harrington 2011). While courtship behaviour and visually-based mate choice does exist in the non-medically important mosquito *Sabethes cyaneus* Fabricius (Hancock *et al.* 1990), vector biologists have long assumed that mating interactions do not occur in medically important mosquitoes such as *Aedes* and *Anopheles*. This notion was probably based on the rapidity with which the mating event occurs. The recent work elucidating male-female mosquito mating interactions suggests that mate choice may exist in these species.

Seminal fluid proteins and their effect on females

Males in many species of arthropods transfer a large number of seminal fluid proteins and peptides (generally referred to as Acps) to females in addition to sperm that can induce a wide range of behaviours (Gillott 2003, Wolfner 2007). Previous experiments in *Ae. aegypti* and *An. gambiae* established the role of male accessory gland extracts in female sexual refractoriness (Fuchs *et al.* 1968, 1969, Fuchs and Hiss 1970, Shutt *et al.* 2010), and the role of accessory gland secretions on fertility in *Ae. aegypti* (Adlakha and Pillai 1975). In addition, blood digestion, ovarian development, oviposition, and feeding behaviour are all influenced by female mating status in *Ae. aegypti* (Downe 1975, Edman 1970, Klowden and Chambers 1991, Klowden and Lea 1979, Lavoipierre 1958, Leahy and Craig 1965). In *Drosophila* a small molecule known as 'sex peptide' temporarily reduces female sexual receptivity after mating (Chapman *et al.* 2003, Chen *et al.* 1988, Wolfner 2007). It is still not clear which specific seminal fluid peptide(s) or protein(s) are associated with monandry in mosquitoes. Recently, a large number of putative Acps have been identified in *Ae. aegypti* (Sirot *et al.* 2008) and *An. gambiae* (Dottorini *et al.* 2007, Rogers *et al.* 2009). Transfer of many *Ae. aegypti* Acps to females during mating has now been demonstrated (Sirot *et al.* 2011). In *An. gambiae,* one specific seminal fluid protein (i.e. a transglutaminase) is responsible for the formation of the mating plug which plays a key role in subsequent sperm storage (Rogers *et al.* 2009). Thus, seminal fluids can have profound effects on females and represent key targets for influencing vector control and potentially vector-borne disease transmission. One study in *Ae. aegypti* demonstrated that females mated to seminal fluid depleted males had a reduced longevity compared to females mated to virgin males, suggesting that certain proteins in the male ejaculate may contribute to female life span (Helinski and Harrington 2011).

Female polyandry

Even though Acps induce monandry in females, low levels of polyandry (<4%) were observed in natural populations of some anopheline mosquitoes (Tripet *et al.* 2003, Yuval and Fritz 1994), but not in others (Yuval 2006). A semi-field cage study with *Ae. aegypti* reported moderate levels (i.e. 14%) of polyandry (Helinski *et al.* 2012). However, whether polyandry occurs in natural free-flying populations of *Ae. aegypti* remains to be determined. Although polyandry is not a prerequisite for effective genetic control strategies (Curtis 1985), knowledge of the rate of polyandry in natural populations is important in order to understand and model gene flow patterns (Magori *et al.* 2009) and interpret data collected during genetic control interventions.

Male reproductive success

Sexual maturation, dispersal and survival

After eclosion, a male mosquito must live long enough to mature and find a mate. Male sexual maturation takes approximately 24-48 h in the majority of species (Clements 1999), and is temperature-dependent. Within this time, male terminalia rotate 180°, and antennal fibrillae needed to hear and locate female wing beat frequencies mature (Howell and Knols 2009, Roth 1948).

Mosquito dispersal is likely influenced by numerous factors such as terrain, availability of resting places, vegetation, host and oviposition sites, and wind direction (Service 1997). The knowledge that exists on mosquito dispersal and life span in the field comes from mark-release-recapture (MRR) studies, although the large majority of these studies were performed with females only (see Service 1993 for a review of the literature). Reports of the maximum flight distance recorded for *Ae. aegypti* males varies, but in general was between 100-800 m (Harrington *et al.* 2005, Muir and Kay 1998, Service 1993). *Anopheles* males dispersed further than *Ae. aegypti,* and maximum flight distances between 0.8-4.5 km were recorded (Service 1993), with a mean flight range of 800 m in a study with *An. gambiae* (Gillies 1961). MRR studies require the ability to recapture released males. While *Ae. aegypti* males can readily be collected indoors and around houses using backpack aspirators, the resting places of some exophilic male anophelines are not well-known. Sampling methods for outdoor resting populations include vegetation collections, and the employment of various resting shelters including resting boxes and pit shelters dug into the ground (reviewed by Service (1993)). Clay pots distributed outside houses were successful in attracting resting populations of male and female *An. gambiae, An. arabiensis, Anopheles funestus* Giles, and *Culex* spp. in Western Kenya (Odiere *et al.* 2007).

Besides dispersal, survival of males is of obvious critical importance to their reproductive success. Little data exists on field survival of males. Most studies rely on naïve log linear estimates that are not entirely appropriate as they assume age-independent survival trends (Buonaccorsi *et al.* 2003). Daily probability of survival obtained from MRR studies for male *Ae. aegypti* were estimated between 0.54-0.70 in Australia (Muir and Kay 1998), and 0.72 in Thailand (Sheppard *et al.* 1969), while for male *Ae. albopictus* released in La Reunion a survival rate of 0.95 was observed (Lacroix *et al.* 2009). A non-linear regression approach supported by bootstrapping estimated rates of 0.37-0.54 for male *Ae. aegypti* from Thailand (Buonaccorsi *et al.* 2003). Log-linear estimates of survival rates for male *Anopheles* were approximately 0.68 for *Anopheles culicifacies* Giles (Reisen *et al.* 1980), and between 0.74-0.93 for *An. stephensi* in Pakistan (Reisen and Aslamkhan 1979). Accurate

estimates of male survival and dispersal are rare and more research is required for candidate mosquito species and geographic strains targeted for genetic vector control.

The effect of body size, age, and nutrition on male mating success

Body size is an important fitness parameter in mosquitoes, and larger female body size is associated with greater fecundity in *Anopheles* and *Aedes* (Briegel 1990, Lyimo and Takken 1993). Male body size played a significant role in *Anopheles freeborni* Aitken mating fitness (Yuval *et al.* 1993) and large males swarmed more often, and more during peak swarming times, than small males. In addition, larger males enjoyed greater mating success than smaller ones (Yuval *et al.* 1993). A small number of studies were conducted with other anophelines, where no such relationship between body size and mating success was observed (Charlwood *et al.* 2002a, Charlwood *et al.* 2003). Body size affects male sperm capacity. Data collected for *Ae. aegypti* has demonstrated a positive correlation between body size and total sperm number (Ponlawat and Harrington 2007), and the number of sperm transferred to females during mating (Ponlawat and Harrington 2009). In addition, small *Ae. aegypti* males experienced more rapid semen depletion than large males when mating with females within an 8 h interval (Helinski and Harrington 2011).

In addition to body size, age is an important factor that influences male mating success. 7-day old *An. gambiae* and *An. arabiensis* males had increased mating success compared to younger males (Verhoek and Takken 1994). Similar effects of age were observed in *An. culicifacies* (Mahmood and Reisen 1994) and *Ae. aegypti* (Ponlawat and Harrington 2009). In contrast to long standing reports (Clements 1999), males continue to mature sperm after eclosion, and sperm numbers increased with age in *An. arabiensis* (Helinski and Knols 2009), *An. gambiae* (Huho *et al.* 2006), and *Ae. aegypti* (Ponlawat and Harrington 2007).

Sugar (i.e. nectar and honeydew) serves as the only nutrition available for adult males. *Anopheles* males sustain their energy reserves by obligatory sugar feeding (Foster 1995), and sugar deprived *An. gambiae* males were not able to inseminate females in large indoor enclosures (Gary *et al.* 2009). Swarming consumed half the energy reserves of male *An. freeborni*, and males sugar fed during the night after swarming (Yuval *et al.* 1994). While *Anopheles* males depend on sugar for their survival and mating ability, only 11% of field collected *Ae. aegypti* males in Thailand (Spencer *et al.* 2005) and 29% in Puerto Rico (Costero *et al.* 1998) had fructose levels high enough to indicate recent sugar feeding activity suggesting that sugar feeding does not occur daily for this species in some dengue endemic regions. Larval conditions can also influence male mating success. A study performed in *An. gambiae* showed that males reared under low crowding conditions as larvae were more likely to succeed in acquiring the first female during mating compared to males reared at high crowding conditions, even though males were similar in body size and teneral reserves (Ng'habi *et al.* 2005).

Male fitness and challenges for future releases

In this section we will provide an overview of fitness studies performed with transgenic mosquitoes and discuss the various challenges released males may face in nature.

Fitness of genetically modified or sterile mosquitoes

SIT studies often determine mating competitiveness, which is assessed by the introduction of sterilised and wild-type males at different ratios in a cage to compete for mates. The proportion

of females inseminated by either male type allows for estimates of mating competitiveness. SIT programs using high radiation doses to sterilise males often report large reductions in mating competitiveness (see Helinski *et al.* (2009) for overview of *Anopheles* radiation studies). The use of lower, semi-sterilising doses in recent years is advocated to increase male fitness (Helinski and Knols 2008, Helinski *et al.* 2009, Parker and Mehta 2007). Other approaches, such as those with *Wolbachia* infected *Ae. polynesiensis* males, found no competitive reduction compared to field collected males when tested in semi-field enclosures (Chambers *et al.* 2011).

Fitness of transgenic lines is often assessed by following the frequency of the transgene over multiple generations which incorporates both the fitness of the transgene itself and the transgenic line (Catteruccia *et al.* 2003, Moreira *et al.* 2004), but other assessments such as survivorship, body size, longevity, and fecundity have been utilised (Irvin *et al.* 2004, Santos *et al.* 2010). For a detailed review of these studies see Marrelli *et al.* (2006) and Scolari *et al.* (2011). The majority of these experiments were conducted in the laboratory, and often with strains not under consideration for release with some exceptions (e.g. RIDL strain). Thus, these findings may not be useful in predicting fitness of potential new release strains. Not too surprising, results varied greatly among the different lines, species, and traits assessed (Marrelli *et al.* 2006). No significant reduction in a number of fitness parameters for transgenic lines relative to non-transgenic control lines was observed in *An. stephensi* (Amenya *et al.* 2010, Marrelli *et al.* 2007, Moreira *et al.* 2004). In one report, transgenic *An. stephensi* mosquitoes resistant to *P. berghei* infection had a fitness advantage over non-transgenic mosquitoes when maintained on infected blood (Marrelli *et al.* 2007). Sustained releases of a RIDL *Ae. aegypti* strain were successful in eliminating wild-type populations when tested in large laboratory enclosures (Wise de Valdez *et al.* 2011). However, other studies did not observe a favourable or neutral effect, and transgenic mosquitoes had reduced fitness compared to non-transgenic mosquitoes in study with *Ae. aegypti* (Irvin *et al.* 2004), and in several transformed lines of *An. stephensi* (Catteruccia *et al.* 2003, Li *et al.* 2008). Few studies have looked at larval fitness of transgenic lines (Bargielowski *et al.* 2010, Irvin *et al.* 2004, Koenraadt *et al.* 2010). In one study, recent field-collected mosquitoes had greater larval performance and teneral energy reserves when competing against inbred or transgenic strains of *Ae. aegypti*, and this effect was exacerbated under low food conditions (Koenraadt *et al.* 2010). Another study reported no consistent trends in larval development times for transgenic versus wild-type laboratory strains of *Ae. aegypti* (Irvin *et al.* 2004), while transgenic larvae of the RIDL OX513A line showed a moderately reduced larval survival and one-day faster development to pupation compared to a wild-type strain (Bargielowski *et al.* 2010).

Reduced fitness of transgenic lines can be explained by a number of reasons including a burden from the transgene product, disruption of gene function, and inbreeding effects (Marrelli *et al.* 2006, Scolari *et al.* 2011). The use of strains containing docking sites such as phi C31 allow for the stable integration of transgenes into a specific place in the genome. Such docking sites have been created in *Ae. aegypti* (Franz *et al.* 2011, Nimmo *et al.* 2006), *Ae. albopictus* (Labbe *et al.* 2010), and *An. gambiae* (Meredith *et al.* 2011). Development of these strains allows for the comparison of transgenes in the same genomic environment, and because the inserted transgene is immobilised, they allow for safe testing under field conditions (Schetelig *et al.* 2009).

Mass production and release

Transgenic or sterile genetic control approaches require the production of large numbers of insects for release. Apart from direct effects of genetic manipulation; colonization, inbreeding, and mass production will likely affect insect fitness and behaviour (Benedict *et al.* 2009, Boller

1972). Colonization and insect rearing conditions are different compared to ambient conditions observed in the field, where insects are exposed to a range of fluctuations in temperature and humidity, availability of food sources, natural lighting conditions, predators, etc. Behavioural changes due to the rearing process were observed in a study performed in *An. quadrimaculatus*, where sterile colony males were far less mobile when released in the field compared to recently colonised sterile males (Dame *et al.* 1964). An interesting example of the importance of larval diet comes from a study in *Culex tarsalis* Coquiilet, where the flight ability of laboratory reared individuals was diminished due to a lack of fatty acids in the larval diet (Reisen *et al.* 1982). The addition of fish oil to the diet improved adult fitness and flight performance (Dadd *et al.* 1989).

The preferred developmental stage for release depends on numerous factors including the number of insects to be released, frequency of releases, number of release points, accessibility to release area, and costs. Mosquito stages suggested for release are pupal (because mortality of this stage is low and it allows avoidance of larval competition effects) or adult stages. Container breeding mosquitoes such as *Ae. aegypti* can be distributed in the pupal stage to households, or placed in focal release points in the village. Adults can be released from cages by ground, or potentially distributed by air. For each release methodology, the effect on adult fitness should be quantified such that adjustments to release ratios can be made. Besides life stage, age of release should be considered if adults are released. In a genetic control program conducted in El Salvador release of older adults (2-3 days) caused less population reduction than the release of pupae or younger males (Dame *et al.* 1981) presumably due to higher mortality rates in older mosquitoes. The optimal age for release needs to be determined for each species and weighed against the costs of insect maintenance. It will be of critical importance to understand the dispersal patterns of released mosquitoes such that informed decisions can be made on the number of release points in a given area and the coverage required.

The number of males to be released will depend on the population density among other considerations (e.g. strength of the drive mechanisms for replacement studies, fitness of release strain, etc.). Adult population density can be estimated from MRR studies using the Lincoln Index (Service 1993) or from pupal surveys, however these methods have limitations. Use of multiple capture-recapture approaches and analysis such as the Jolly-Seber model can provide more refined information (reviewed in Service 1993).

Survival, mate location, and successful insemination

Once released, males need to survive long enough to find and inseminate a female. As discussed above, many of the mechanisms for survival or mate location are unknown and thus it is difficult to predict which male characteristics are important for released males to have. Recent advances in understanding male acoustic interactions suggest that harmonic convergence could be important for mate assessment and successful copulation (Cator *et al.* 2009, 2010, Pennetier *et al.* 2010, Warren *et al.* 2009), and this trait could be used as an assessment tool to predict male mating success, although it does not allow for high-throughput male assessment and may be impractical for many programs. Harmonic convergence behaviour can be measured by placing free-flying or tethered males in a mating arena with a tethered female close to a particle velocity microphone. Flight tones of males and females prior to mating are recorded and can be analyzed with appropriate software to determine convergence behaviours (Cator and Harrington 2011, Cator *et al.* 2009, 2010). If modified males are unable to converge, then their probability of mating success in the field might be greatly reduced. Detection of a convergence deficiency would signal the need to enhance diets or consider reconstruction of the modified line.

Males have the resources to mate with multiple females within one day before depletion of sperm or seminal proteins occurs. In *Ae. aegypti*, males could inseminate 4-6 mates before reduced numbers of sperm were transferred (Foster and Lea 1975, Gwadz and Craig 1970, Jones 1973). A recent study reported a reduction in fecundity by more than 50% in females mated to semen depleted males when males became depleted after 3-5 matings for small and large males, respectively (Helinski and Harrington 2011). It is not known, however, how many potential mates a male released in the field will encounter each day or within his lifetime, and it is likely that some males will never mate.

It is important that females do not select against released males. Under some circumstances, behavioural resistance was observed in SIT programs of the melon fly *Batrocera cucurbitae* Coquillett and the Mediterranean fruit fly where females actively discriminated against released males. Female *Ae. aegypti* mosquitoes can actively reject males under laboratory conditions (Jones 1974), and thus there is the potential for rejection behaviour to occur.

After insemination occurs, released males need to induce the same post-mating responses in females as do wild-type males. As discussed above, Acps can affect many aspects of female postcopulatory behaviour, such as her receptivity to a subsequent mating. The novel information gathered on semen protein composition in *Ae. aegypti* and *An. gambiae* (Rogers *et al.* 2009, Sirot *et al.* 2011) will allow for the evaluation of such proteins in modified males. Semen proteins are important for sperm viability and function in other insects (Avila *et al.* 2010). In addition, the ability of females to exercise control over sperm storage and utilization has been demonstrated for other insects (Eberhard 1996), and may be important in mosquitoes.

Recommendations for developing effective genetic control programs

In this section we discuss information required for effective genetic control programs. As discussed previously, two key approaches to genetic mosquito vector control are population replacement and population reduction. While many relevant aspects of fitness are the same regardless of the approach used, in some cases they can be different for population replacement vs. reduction. We have indicated this difference whenever possible below.

Even though initial laboratory assessments are important, we strongly recommend taking experiments to field cages due to unknown environmental influences that can only become apparent in nature. Until more studies are conducted and reported to the greater scientific community, it will be difficult to know what assessments are most important. Ultimately, researchers should decide the optimal balance of laboratory and field cage assessments at the outset of their program given their budget and timeline. Re-evaluation of the balance can then occur throughout the study as unanticipated issues arise. Because considerable time is required to plan, obtain approvals, conduct community engagement, and execute field cage trials, we advocate that researchers start planning this component as early as possible after project initiation.

Information needed for effective genetic control programs: field biology

While more recent information regarding the swarming behaviour of *Anopheles* mosquitoes (Diabate *et al.* 2009, 2011, Manoukis *et al.* 2009) has added to the existing body of literature (Charlwood *et al.* 2002a,b, 2003, Yuval *et al.* 1993), mating biology studies for *Aedes* mosquitoes have scarcely been pursued since the 1970s (Gubler and Bhattacharya 1972, Hartberg 1971), with few recent field observations (Bellini *et al.* 2010, Cator *et al.* 2011). More research is essential and

studies of *Aedes* swarms should be conducted in field settings where observers note the onset, location, and duration of swarm formation. Dispersal and survival are also understudied areas of male biology that require more detailed investigation. It is important that research is conducted in different ecological settings where proposed genetic control programs will be deployed, as variation may occur across a range of geographical and ecological settings. Better methods to estimate population size, age and structure also need to be developed such that decisions can be made on the number of males needed for a release.

From laboratory bench to field application

Assessment of baseline performance levels

After obtaining a transgenic line, basic assessments of strain and male fitness would be performed in the laboratory. These experiments should include determination of strain survival (at egg, larval, and adult stage), and the mating ability of males. Female fecundity after mating will reveal initial problems with male semen. For population replacement strategies, cage experiments should be undertaken to follow the fate of the transgene over a couple generations similar to the ones performed by Catteruccia *et al.* (2003).

Release methodologies such as those employed with certain RIDL strains (Fu *et al.* 2010) resulted in population reduction via late-acting lethality in RIDL male offspring. This approach has an advantage over SIT strategies because offspring compete at larval stages when density-dependent competition may occur (Dye 1984). However, analyses by Legros *et al.* (2009), question the importance of density dependence in early immature stages. Thus, for these strategies, the assessment of larval competitiveness of transgenic larvae in the presence of wild-type larvae needs to be determined. Conditions should be chosen that maximise competition and the use of appropriate controls is important to interpret findings.

Seminal fluid proteins transferred from males to females during copulation have been identified in *An. gambiae* and *Ae. aegypti* (Rogers *et al.* 2009, Sirot *et al.* 2011) and their expression levels can be quantitated using RT-PCR (Thailayil *et al.* 2011) or protein assays such as ELISA or Western blotting. As discussed in the Section 'Mosquito biology', male flight tones can be measured prior to mating to determine if harmonic convergence behaviour, an indicator of male performance, occurs.

For all of the above assessments, it is important that strain characteristics are compared to a wild-type strain consisting of recently collected individuals from the potential release site. A study in *An. gambiae* determined that body size and lipid reserves of wild males were substantially greater than males reared under standard laboratory conditions (Huho *et al.* 2007). This implies that conditions used in the laboratory to rear mosquitoes may not be sufficient to achieve strong fitness potential once released in the wild and that distinct differences exist between laboratory and wild mosquitoes. For example, it is not clear what combination of resources mosquito larvae naturally ingest. Larvae are known to consume a variety of microorganisms and particulate organic detritus (Clements 1999, Merritt *et al.* 1992), but replicating this diet in the laboratory without more information is impossible.

If after the initial assessments the strain does not perform well, testing of candidate strain should end and new lines created. If results are promising, the transgenic line can move to the next step of semi-field cage evaluation.

Semi-field assessments

The next phase of evaluations of transgenic/sterile strains should be conducted in semi-field cages rather than the laboratory as field cages provide more natural conditions such as lighting, temperature and humidity while maintaining containment. Cages should be established that include natural habitat (vegetation if necessary, breeding sites and food sources) of free ranging mosquitoes. Cages should be of ample size. We recommend a minimum size 20 m^3 after cage studies performed in Mexico with *Ae. aegypti* (Facchinelli *et al.* 2011, Figure 1). Larger and more contained semi-field cages such as the ones described by Ritchie *et al.* (2011) are ideal but their high costs will not make them realistically achievable for all research groups. Before releasing transgenic/sterile males, wild-type mosquitoes should be released and monitored in cages to confirm that conditions used are appropriate and that containment is absolute (Benedict *et al.* 2008). It is essential that control cages (wild-type mosquitoes only) are established and evaluated side-by-side with experimental cages in these studies. Low generation virgin wild-type males and females collected from the surrounding area should be used as controls.

For population reduction strategies such as the SIT and some RIDL approaches, male competitiveness can be determined by releasing transgenic/sterile males with wild-type males and wild-type females at a 1:1:1 ratio. Densities used in such studies should reflect the natural density of the species in the release area. Studies with Mediterranean fruit fly programs routinely use a mating competitiveness (MC) value above 0.2 (i.e. released males obtain 20% of the mates when competing with wild-type males for wild-type females at 1:1:1 ratio) (FAO-IAEA-USDA 2003) as acceptable; however, the threshold for mosquitoes is unknown and could be different. Low MC can be overcome by increasing the release ratio of males (10:1:1 or higher), and these experiments can be performed if initial competitiveness is low. However, excessively high release ratios may not be operationally feasible for open field releases. Other experiments that can be performed to

Figure 1. Example of a cost-effective field cage (20 m^3) used in studies performed in Mexico with Aedes aegypti *(Facchinelli* et al. *2011).*

test the fitness of strains include sustained releases of insects to eliminate wild-type populations similar to a study conducted with a RIDL line of *Ae. aegypti* (Wise de Valdez *et al.* 2011).

For population replacement strategies, it will be important to assess the ability of the transgenic strain to drive the transgene into a wild-type population. Such studies follow the frequency of the transgene over multiple generations after an initial introduction into a wild-type population (Catteruccia *et al.* 2003, Moreira *et al.* 2004). Success will depend on the strength of the drive mechanism, the fitness of the males, and the initial release ratio.

If evaluations are favourable, cage assessments can move forward with mass reared males. Mass rearing insects can result in decreased fitness, behavioural changes, or instability of the transgene, thus it will be important to monitor the strains during large scale rearing. Strategies to improve on strain fitness can include addition of different dietary components and holding conditions. Knowledge and guidance on this topic can come from existing fruit fly mass-rearing programs (Cáceres *et al.* 2007, Liedo *et al.* 2007). In addition, continuous introgression of field material can be performed to maintain genetic diversity if required. A potential recent advance to avoid inbreeding effects is the use of genetically diverse laboratory strains (Wise de Valdez *et al.* 2011, Wise de Valdez *et al.* 2010); however the need for maintenance of these strains might not make them practically applicable to large scale release efforts and especially not useful for *Anopheles* vectors as eggs cannot be stored.

Small open field release

If the performance of mass-reared males is good, studies can move forward to a small scale open field release. Any program planning semi-field cage and open field releases must adhere to the legal, social and ethical standards for the particular region where the study is being conducted (Lavery *et al.* 2008). Ideally these studies should be conducted in an isolated setting (small island or community surrounded by barriers to dispersal).

In conclusion, while many challenges remain in the development of genetic vector control strategies, recent advances have highlighted the significant potential of this approach for reducing mosquito populations and, ultimately, human mortality and morbidity from vector borne infections. A greater understanding of mosquito biology, ecology and behavior, especially for males as highlighted here, will be critical for ensuring future success.

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Epilogue

13. Ecology of parasite-vector interactions: expect the unexpected

Constantianus J.M. Koenraadt and Willem Takken

Abstract

As revealed by the previous chapters, a wealth of information has become available on the biology and ecology of parasite-vector interactions. This has led to a greater understanding of the more fundamental aspects on how parasites manipulate their arthropod hosts, as well as on how the arthropod's immune system responds to this. Most interestingly, it has also led to novel applications for sustainable control of vector-borne diseases, such as exemplified by the ongoing field trials with *Wolbachia* as a disease-controlling endosymbiont in *Aedes aegypti* L. as well as by the release of transgenic male *Ae. aegypti* for the control of dengue in Grand Cayman island, Brazil and Malaysia. In this chapter we synthesize the reviewed knowledge and conclude that in many instances environmental and evolutionary forces can pull trade-offs between parasites and vectors in unexpected directions.

Keywords: vector-borne disease, parasites, pathogens, interaction, genetics, immunity, transmission

Introduction

Why are some arthropods such efficient vectors of parasites and others not? Even organisms that look so much alike and are very closely related genetically can differently contribute to the transmission of a parasite. This question has puzzled scientists for decades already. At the turn of the 19th century, the discovery of mosquitoes being the vector of viruses, protozoans and nematodes by eminent scientists such as Major Walter Reed (Yellow Fever), Sir Ronald Ross (malaria) and Sir Patrick Mason (bancroftian filariasis) has been a major turning point. Still, it took another 50 years or so to really understand the epidemiological importance of the ecology of arthropods as vectors of disease. The Ross-MacDonald model (for a historical review, see Smith *et al.* 2012) on the basic reproductive number, i.e. the number of new infections arising as a result of the introduction of an infective case in an immunologically naive population, is still used by academia and has been the starting point for many chapters in this volume.

This book aims to provide the latest insights into the ecology of parasite-vector interactions. And indeed, the various chapters are evidence that the intricate relationship between a parasite and its vector is interwoven in a web of interactions with its internal micro-biome and the external environment. It is clear that various environmental and evolutionary forces can pull in opposing directions, sometimes leading to unexpected outcomes.

Underestimating disease risk

Vectors are poikilothermic ('cold-blooded') and therefore highly vulnerable to variable environmental conditions. Only recently these effects have been explored in more detail. Temperatures vary considerably throughout the day, are different for indoor as compared to outdoor mosquito populations and different for the aquatic environment in which the larval stages of mosquitoes develop. Paaijmans and Thomas (Chapter 5) have explored these effects on mosquito and *Plasmodium* development and conclude that a comparison of linear versus non-linear models demonstrates that the standard use of the mean outdoor temperature in malaria

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risk models is not able to capture important features of the actual microclimate experienced by mosquitoes. In fact, ignoring diurnal temperature fluctuation in modelling may underestimate malaria risk in cooler areas such as African highlands (Paaijmans *et al.* 2009).

Size matters

That the aquatic environment of mosquitoes is of importance for the actual transmission of associated parasites or arbo-viruses is also concluded by Alto and Lounibos (Chapter 4). In these water bodies, the larvae compete for food either with their conspecifics or with larvae from different, and perhaps invading species, such as the tiger mosquito *Aedes albopictus* Skuse (Chapter 14, Juliano and Lounibos 2005, Takken and Knols 2007). These interactions have an impact on the size of the emerging adult. Various studies have demonstrated that smaller adults are more susceptible to arbo-viruses due to their thinner midgut basement membrane, thereby weakening the midgut escape barrier for viral particles. However, other studies revealed that, for example large *Aedes triseriatus* Say are more susceptible to La Crosse Encephalitis virus (LACV) than smaller ones (reviewed in Chapter 4). Probably larger adults ingest more virus particles in blood meals, thereby increasing their chances of becoming competent vectors later in life. It remains unclear what the precise physiological mechanisms are that determine the outcome of these interactions, although important advances have been made in our understanding of the expression of immunity related genes (Chapter 2). As soon as a mosquito is challenged with a pathogen, hundreds of genes are up- or down regulated with the aim to mount a neutralizing immune response. It is generally thought that there is a trade-off between this immune response and fitness of the vector: on the one hand invading pathogens can negatively affect vector survival, while on the other an immune response can be energetically expensive and thus also lead to a reduced input into reproduction. This has important implications for the development of transgenic vectors, especially those designed to express a pathogen-killing effect upon feeding on an infectious blood meal (Chapters 2 and 12). However, a moderate fitness cost resulting from increased immune activation may be acceptable since it may offset the fitness cost of the actual infection. The general consensus is that transient expression of a transgene in a tissue and stage specific manner can limit the negative effects of an over-expression of immune genes and, if the refractoriness gene is linked to a strong gene driver, this method can be a viable alternative for disease control (Chapter 2). If that goal can indeed be achieved in the long run needs to be addressed from an evolutionary perspective. Lambrechts and Koella (Chapter 6) argue that quantitative estimates for malaria parasite-induced changes of the parameters making up the basic reproductive rate should be obtained under more realistic field conditions. A decreased efficacy of a transgenic malaria control programme could be the result of a greater investment of the malaria parasite into evading or suppressing the refractoriness mechanism inside the mosquito. This requires an integrated research programme on experimental evolutionary ecology and mathematical modelling (Chapters 6 and 10).

Sugar feeding

Sugar feeding by mosquitoes is generally considered to be required for body maintenance and providing energy for flight (Takken and Knols 1999). Hence, without sugar the mosquito's survival and thus lifetime reproductive capacity is significantly reduced. This has of course also a major impact on the likelihood of transmission of the malaria parasite. However, when mosquitoes choose wisely from the available nectar sources (flowers, honeydew and extra-floral nectaries), they are even capable of clearing a malaria infection, as a result of the inhibitory factors in plant nectar (Chapter 3). In other words, the spatial and temporal distribution of plants in a specific

environment may have an important impact on malaria transmission. According to Stone and Foster (Chapter 3), this knowledge could be exploited by developing plant-volatile baited traps, as these target a much wider segment of the mosquito population than is typically sampled with CO_2- baited traps and ovitraps.

The role of parasite and vector secretions

Though much research on parasite-vector interactions focuses on the role of mosquitoes and their associated pathogens, there are many parallels with other vector-parasite systems, such as tick-*Borrelia* (Chapter 7) and sandfly-*Leishmania* (Chapter 9) interactions. Both *Borrelia* bacteria and *Leishmania* protozoa are capable of manipulating their host in order to enhance their own transmission. Both chapters highlight the unexpected, but important role of secretions in the actual transmission, such as tick saliva in the case of *Borrelia* and a gel secreted by the promastigote form of *Leishmania*. A protein in tick saliva (Salp15) binds to *Borrelia* bacteria and suppresses a T-cell mediated immune response in the host , thereby providing a protective coat for the bacteria. In *Leishmania* it has been established that the promastigote secretory gel, which mostly contains filamentous proteophosphoglycans, causes exacerbation of disease symptoms in mice.

Microbiome interactions

Besides these secretions originating from either the vector or the pathogen itself, the microbiome that is present in the vector plays an important role in determining the outcome of pathogen-vector interactions. Several tens of bacteria species have been identified in the mosquito midgut that are thought to have an effect on digestion, nutrition and reproduction (Ramirez *et al.* 2012). The most well-known example is the *Wolbachia* bacterium, that was first identified from the reproductive tissue of *Culex pipiens* Say. Its effects on arthropod reproduction through feminization, male killing, parthenogenesis and cytoplasmic incompatibility have puzzled scientists for a long time. This has triggered scientists to think about the possible role of *Wolbachia* for vector control, either as a driving mechanism for transgenic elements that block reproduction of a pathogen (see also Chapter 12) or as direct control agent itself. The latter option came rather unexpectedly: introduction of specific *Wolbachia* strains into the dengue vector *Aedes aegypti* L. resulted in a shortened life-span of the mosquito as well as to the interruption of transmission, because the presence of *Wolbachia* reduces the replication of dengue virus (Chapter 8). One appreciates these findings even more considering that it took the researchers more than three years of continuous serial passage in mosquito cell cultures to adapt the *Wolbachia* strain to these cell lines before they were able to inject it successfully into *Aedes* mosquitoes. The most exciting news is that currently trials are underway to evaluate the impact of *Wolbachia* on mosquito population dynamics and dengue transmission in the field (Hoffmann *et al.* 2011).

Informed decisions for disease control

As demonstrated in the first chapters, the outcomes of parasite-vector interactions can be unexpected at times. With the recent advances in molecular biology and tools to analyse high-throughput data, we now have a more solid understanding of these interactions in a relatively complex environment. Still, decision makers and other stake-holders are eventually most interested in what this means in terms of investing in novel vector control tools or how they can distribute their limited resources more efficiently (Woolhouse *et al.* 1997). In case of malaria for example, a targeted malaria control approach is estimated to have a four-fold higher impact than an untargeted approach (Chapter 11). This is due to the fact that some areas (households,

villages or regions) experience a higher transmission intensity compared to the average value of the larger area. Such heterogeneities, or hotspots of transmission, may fuel malaria transmission in wider regions. Identification of such hotspots is therefore of crucial importance, and efforts are underway to identify the most reliable indicators based on serological markers of malaria exposure (Chapter 11). Transmission models are very useful in this regard, as they are able to incorporate the multiple interactions and address the outcome on the longer term. This can be done by using a backward or forward modelling approach, whereby the first aims to interpret complex mosquito data and the latter is able to predict the consequences of specific interventions (Chapter 10). For example, according to Ace and Hancock (Chapter 10) introducing fungal biopesticides as a novel tool to disrupt *Anopheles-Plasmodium* interactions has a variable impact depending on the locally prevailing climate. Counter intuitively, the largest impact of fungal biopesticides on entomological inoculation rate, a measure of malaria transmission intensity, is achieved at cooler rather than at warmer temperatures. This is probably due to the longer time that is available for the infection process of the fungus.

Conclusions

One thing has clearly surfaced throughout the various chapters of this book: we need to stay one step ahead of parasites if we are to control them in the end. To expect the unexpected requires innovative thinking and focussing on research areas that are currently under-studied. For example, because female mosquitoes are the agents that transmit the malaria parasite, little attention has been paid to the biology of male mosquitoes (Ferguson *et al.* 2005). However, for the development of genetic control strategies, the fitness of transgenic males that are to be released *en masse* is crucial for the success and sustainability of a genetic control programme (Chapter 12). In addition, the continuous evolutionary arms race between parasites, vectors and our control efforts may lead to the development of more virulent strains or to parasites being no longer susceptible to the commonly used drugs (Smith and Schapira 2012). This does not happen everywhere at the same pace, but is very heterogeneous in space and time. Understanding parasite-vector interactions in such an ecological context remains challenging, but highly rewarding when they lead to new interventions that make our lives healthier and happier.

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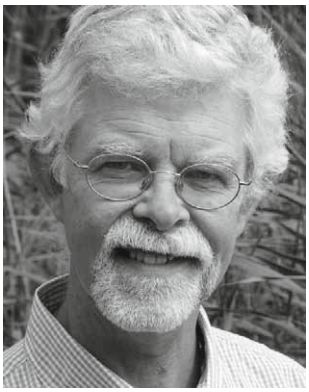
About the editors



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